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I am submitting herewith a thesis written by Yolanda F. Kirkpatrick entitled "Molecular Approaches in Attempts to Identify the Flagellin b-type Gene in Clone pKW52." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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Molecular approaches in attempts to identify the flagellin b-type gene of *Pseudomonas aeruginosa* strain PAO1 in clone pKW52

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Yolanda Faye Kirkpatrick
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DEDICATION

This thesis is dedicated to the other Yolandas that have paved the way and the Yolandas that are to come!

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There are many people I would like to thank for their unwavering support and encouragement...for they also deserve their names on this thesis. Mother thank you for insisting that I go to college, because yes "there is so much for you (me) to learn." Adrienne thank you for never allowing me to lessen my standards. Patrick thanks for keeping me updated on the latest football scores. Suzanne, I appreciated your benchtalks and the interesting football Sundays. Thanks Elizabeth and Jack for recognizing that I needed a ski trip! Andrenette and Angie I will not forget the lunch getaways. Kumar and Gene I value your insight and guidance. Finally, I want to thank Dr. Mark Seyferd for taking a chance and giving me my first real job, and starting the intellectual spark by encouraging me to "wonder why things happen."

ABSTRACT

Pseudomonas aeruginosa is an important pathogen for susceptible individuals particularly those immunocompromised due to the prolonged use of drugs, noscomial infections, burns, or cancer. In addition, *P. aeruginosa* infections are high among cystic fibrosis patients. *P. aeruginosa* presents a unique health concern and challenge for researchers due to its multiple virulence factors and the increasing number of patients developing *P. aeruginosa* infections. *P. aeruginosa* produces a variety of toxins and enzymes. The flagellum of *P. aeruginosa* allows the bacterium to be motile. Motility has enabled the *P. aeruginosa* bacterium to rapidly colonize the host's body.

The major structural component of the *P. aeruginosa* flagellum is the protein, flagellin. *P. aeruginosa* flagellin was categorized two major groups, designated *a* and *b* via slide and tube agglutination assays and immunofluorescence technique. The *a* -type flagellin is composed of a major a_0 antigenic component and one to three subantigen types, a_1, a_2, a_3, and a_4. The a-type flagellins have molecular weights of 45,000 to 52,000. The *b*-type flagellin appear to be antigenically homogenous and have a molecular weight of 53,000. It is the centermost region of the flagellin that is antigenic and highly variable even within one species.

Oligonucleotides 1N and 1C, derived from the N-terminal and C-terminal nucleotide sequence of the cloned *P. aeruginosa* (strain PAK) flagellin a-type gene, annealed to clone pKW52 (flagellin b-type) and an approximately 600bp fragment was amplified by PCR. The 600bp fragment was cloned in vector pCRII, and a partial nucleotide and amino acid sequence of the resulting clone pKWII was determined. Southern hybridization with radiolabeled probes was used in attempts to locate and identify the flagellin b-type gene in pKW52 and pKWII, respectively. The 1.7kb flagellin (a-type) gene probe did not hybridize to pKW52 and pKWII under low and high stringency hybridization conditions. In immunological studies of pKW52 flagellin antigen expression was detected in *E. coli* cells harboring clone pKW52. Flagellin expression was not detected in *E. coli* cells harboring clone pKWII.

The results of this thesis research suggest: 1) based on Southern hybridization experiments the sequence identity between the PAK 1.7kb flagellin a-type gene and clone pKW52 is estimated to be less than 90%, 2) the expression of flagellin b-type protein in *E. coli* cells harboring pKW52 suggested that clone pKW52 may contain the flagellin b-type gene, 3) colony immunoblotting experiments with antiflagellin antibodies correlated with Southern hybridization results demonstrating that pKWII did not contain the flagellin b-type gene, and 4) PCR and Southern hybridization experiments are molecular biology techniques that may be used to locate the flagellin b-type in clone pKW52.

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CHAPTER 1

INTRODUCTION

Characteristics.

Pseudomonas aeruginosa is a Gram-negative, saprophytic rod that is widely distributed in soil, water, sewage, the mammalian gut, and plants (Gilardi, 1985). This aerobic organism can utilize a variety of sugars, fatty acids, dicarboxylic and tricarboxylic acids, alcohols, polyalcohols, glycols, and aromatic compounds (Brock *et al.*, 1984). *P. aeruginosa* varies in length from 1 to 3 μm and from 0.5 to 1.0 μm in width. It possesses a glycocalyx and is mobile with a single polar flagellum (Doggett, 1979). *P. aeruginosa* is an opportunistic pathogen of humans, certain animals, insects, and plants (Gilardi, 1985). For humans, it is an especially important pathogen for burn, cancer and Cystic Fibrosis (CF) patients (Froland, 1981). *P. aeruginosa* infection is generally restricted to hospitalized patients who become susceptible as a result of surgical procedures, inhalation therapy, chemotherapy and urinary catherization (Scheld *et al.*, 1991; Gilardi, 1985). In addition, the prolonged usage of immunosuppressive agents, corticosteroids, antimetabolites, antibiotics, and radiation treatments predisposes patients to *P. aeruginosa* infection (Gilardi, 1985). *Pseudomonas* causes pulmonary, urinary tract, bone and joint infections, malignant external otitis, endocarditis, meningitis, and bacteremia. *P. aeruginosa* is frequently not the initial pathogen of these diseases and the incidence of *Pseudomonas* as a cause of these infections varies upon the clinical situation (Kohler *et al.*, 1979).

Pathogenesis and Virulence.

P. aeruginosa has a multifactorial pathogenesis which may be due to the variety of toxins and enzymes that it produces. *P. aeruginosa* virulence-associated factors are lipopolysaccharide, mucopolysaccharide capsule, pili, proteases, phenazine pigments pyocyanin and rhamnolipid, phospholipase C, siderophores, exotoxin A, exoenzyme S, and leukocidin. The lipopolysaccharide (LPS) of this bacterium is relatively less toxic than the LPS of other selected bacilli, but it serves as a proximal mediator for a great variety of inflammatory events (Fick, Jr., 1993). The mucoid exopolysaccharide (MEP) which is composed of mannuronic and guluronic acids with O-acetylation results from the exuberant production of mucoid (Speert, 1993). MEP suppresses neutrophil and lymphocyte functions including phagocytosis, neutrophil locomotion, and lymphocyte transformation. Also, mucoid strains have a propensity to persist in the respiratory tracts of CF patients. The reasons for the persistence of mucoid *P. aeruginosa* in the respiratory tract of CF patients is unclear. One explanation is that mucoid *P. aeruginosa* adheres to the respiratory tract epithelium and mucin of CF patients. Another explanation is that the antiphagocytic and immunosuppressive properties of MEP allow the bacterium to evade host defenses (Mai *et al.*, 1993). Pili are another surface component synthesized by *P. aeruginosa* that contribute to its pathogenicity. The pili play a key role in the attachment of the bacterium to host cell receptors (Ishimoto *et al.*, 1992).

P. aeruginosa produces two proteases, alkaline and neutral metalloproteinase (Fick, Jr., 1993). These proteases damage the lung through the digestion of extracellular matrix and direct cytotoxicity. *In vitro* data suggest that these proteases may also contribute to lung injury in CF patients via their ability to cleave the host iron-binding proteins transferrin and lactoferrin (Britigan *et al.*, 1993). Furthermore, *P. aeruginosa* siderophores pyoverdin and pyochelin make iron more available for bacterial growth and metabolism and contribute to the generation of toxic oxygen free radicals (Britigan *et al.*, 1993; Fick, Jr., 1993).

This bacterium is easily recognizable on nutrient agar for its characteristic pigmentation. Of the 32 bacterial species producing phenazine pigments, *P. aeruginosa* is the only clinically significant bacterium for which the production of phenazine pigments is a distinctive feature. "The phenazines are low molecular

weight compounds containing a three-ringed, heterocyclic nucleus with nitrogen substitution at the center positions of 5 and 10" (Sorensen *et al.*, 1993). These pigments contribute to ciliary disruption and provide strong reducing potential (Sorensen *et al.*, 1993).

All virulent strains of *P. aeruginosa* produce heat-labile phospholipase C. The combination of phospholipase C with extracellular toxins produced by this bacterium could cause considerable cytopathology (Vasil *et al.*, 1982). The exotoxins of *P. aeruginosa*, exotoxin A and exoenzyme S, are ADP-ribosyltranferases that inhibit protein synthesis of susceptible cells (Fick, Jr., 1993; Frank *et al.*, 1994). *P. aeruginosa* produces a 27-kD protein, leukocidin, that is reactive against leukocytes of different animal species (Scharmann, 1976). Leukocidin prevents leukocyte motility and phagocytosis, and impairs bactericidal capacity (Scharman *et al.*, 1976; Baltch *et al.*, 1985; Kluftinger *et al.*, 1989).

The flagella of *P. aeruginosa* provide motility and serve as antigenic determinants for host immune responses; however, the flagella are also an important factor in this organism's pathogenicity. The role of flagella, whether functional or nonfunctional, is associated with virulence (Holder, 1993). Results obtained from the burned-mouse model showed that motile strains of *P. aeruginosa* rapidly invade and colonize the host's body. In comparison, isogenic nonflagellated strains had a considerable loss of virulence (Drake *et al.*, 1988). Even though motility is an important virulence factor of *P. aeruginosa*, the nonfunctional flagella play a role in *Pseudomonas aeruginosa* infections. *P. aeruginosa* mucoid strains contribute to virulence via bacteria-induced injury of the host. The MEP-coated *P. aeruginsoa* cells evade phagocytic killing and are able to multiply in large numbers in the host. The diffusion of Pseudomonal toxins, enzymes, and metabolites damages the host tissues. The diffusion of phagocyte-derived toxic enzymes and free radicals also causes inflammatory damage. The host's defenses are unable to eliminate the mucoid cells. Therefore, the cells continue to multiply in large numbers and the cycle of bacteria-induced inflammatory damage persists (Holder, 1993; Mai *et al.*, 1993).

Flagellum Structure.

Procaryotic cells have different patterns of flagellation. The pattern may be peritrichous, or single or multiple flagella may be at one or both ends of the cell. Other flagellated cells may possess numerous lateral flagella. Furthermore, the flagella of some cells are located entirely in the periplasmic space between the inner and outer membranes. The general structure of the procaryotic flagellum consists of three sections, a filament, a hook, and a basal body. The flagellar filament is a helical thread typically 5 - 10 µm long with an outer diameter of about 20 nm (Jones *et al.*, 1991). In most bacterial species the major component of the flagellar filament is the protein flagellin (Macnab, 1987; Iino, 1985). *P. aeruginosa* flagellin was categorized via slide and tube agglutination assays and immunofluorescence technique to be of two major groups, designated *a* and *b*. The *a* flagellin is composed of a major a_0 antigenic component and one to three of subantigen types, a_1, a_2, a_3, and a_4. The a-type flagellins have molecular weights of 45,000 to 52,000 (Anderson *et al.*, 1989). The b-type flagellin appear to be antigenically homogenous and have a molecular weight of 53,000 (Allison *et al.*, 1985). It is the center region of the flagellin that is antigenic and highly variable even within one species (Macnab, 1987; Vonderviszt *et al.*, 1991). The second section of the flagellum is the hook. The hook is a curved rod structure about 50 nm long with a diameter of about 20 nm (Jones *et al.*, 1991). It provides attachment for the filament to the cell base and stability for the filament structure (Wagenknecht *et al.*, 1982; Silverman *et al.*, 1977; Iino, 1985). The hook is composed of a single kind of protein called the hook protein (Iino, 1985). The third component of the flagellum, the basal body, is a multiring structure connected to the hook by a rod (Cohen-Bazire *et al.*, 1967). This rod passes through the centers of the four rings, L, P, S, and M and a cylinder fills the space between the L and P ring. The L ring is the outermost ring and it corresponds to the lipopolysaccharide membrane of the cell surface. The M ring is the innermost ring and it corresponds to the cytoplasmic membrane. The P ring lies in the peptidoglycan layer and the S ring in the periplasmic space (Iino, 1985). In addition, the basal body is a key part of the flagellar motor allowing flagella rotation. This rotation, clockwise or counterclockwise, is powered by the flow of protons across the cytoplasmic membrane (Jones *et al.*, 1991).

Flagellum Assembly.

The bacterial flagellar filament can self-assemble *in vitro* from flagellin monomers. Assembly *in vitro* has been described as crytallization and can be induced by adding short fragments (seeds) of filament or by adding a precipitant such as ammonium sulfate or polyethylene glycol to the culture. Assembly of flagellin *in vivo* occurs with the transport of synthesized flagellin monomers from the cytoplasm to the tip of the hooks. At the tip of the hooks polymerization of flagellin starts, and is accompanied by its conformational change through interaction with one or more hook accessory proteins. Elongation of the filament proceeds in this manner with the most recently incorporated flagellin molecule acting as part of the nucleus for polymerization of the next flagellin monomer to arrive. *In vitro* and *in vivo* elongation of the flagellar filament occurs at the distal end of the filament (Iino, 1985; Jones *et al.*, 1991).

Flagellum Genetics.

Approximately 40 genes are involved in the flagellar and motility systems of *Escherichia coli* and *Salmonella typhimurium*. The flagellar motor and chemotaxis genes (except some of the chemotactic receptor genes) comprise about 13 operons clustered in Regions I, II, and III of the chromosome. In addition, *Salmonella spp.* have a Region IV that contains genes involved in phase variation (Jones *et al.*, 1991). The genetic makeup of *P. aeruginosa* flagella is thought to be similar to the genetic makeup of *E. coli* and *S. typhimurium* flagella (Iino *et al.*, 1988). In *P. aeruginosa*, genetic mapping has shown the flagellar genes to be in Regions I and II of the chromosome. Region I contains one motility and five flagella cistrons. Region II contains ten flagella and two chemotaxis genes (Tsuda *et al.*, 1983).

Related Experiments.

The type a flagellin gene of *Pseudomonas aeruginosa* strain PAK has been cloned, sequenced, and the transcriptional start site of the structural gene

determined (Totten *et al.*, 1990). The regulation of flagellin expression in *P. aeruginosa* has been determined. The fliA (rpoF) gene of *Pseudomonas aeruginosa* encodes an alternative sigma factor required for flagellin synthesis (Starnbach *et al.*, 1992). The transcription of the flagellin gene by s^{28} (rpoF) is mediated by s^{54} (rpoN) (Starnbach *et al.*, 1992). RpoN transcribes the regulatory protein flaR which binds to an upstream activating sequence activating flagellin transcription. Furthermore, alignment of the N-terminal 150 amino acids and the C-terminal 80 amino acids of *P. aeruginosa* PAK flagellin with the counterpart regions of *Bacillus subtilis, Escherichia coli, Campylobacter crescentus, Borrelia burgdorferi,* and *Campylobacter coli* showed that the flagellin amino acid similarity among these genera ranged from 53 - 75%. Southern hybrization findings indicate the presence of one copy of the flagellin gene in *P. aeruginosa* PAK (Totten *et al.*, 1990). This and other genetic information will provide a basis for the study of *P. aeruginosa* strain PAO1, b-type flagellin.

Investigations of *P. aeruginosa* strain PAO1 began with the isolation and cloning of the flagellin b-type gene by Kimberly Kelly-Wintenberg. The tri-parental mating procedure was used to mate the PAO1 genome into the flagellin (-) mutant *P. aeruginosa* strain PA103 (Kelly-Wintenberg *et al.*, 1989; Ohman, 1986). The entire genomic bank was screened and isolates that showed more than 80% of the motility and produced a single-polar flagellum typical of the wild-type PAO1 were retained. Plasmids from PA103 designated pKW3, pKW8, pKW23, pKW52, pKW84 and pKW333 were transformed in *E. coli* HB101 cells. The transformed *E. coli* HB101 cells were isolated by their ability to grow in the presence of tetracycline (300 mg/ml) and anaerobically. To detect the production and expression of *P. aeruginosa* b-type flagellin in the *E. coli* HB101 cells colony immunoblots using b-type polyclonal antiserum were performed. The *E. coli* HB101 cells transformed with these pKW clones produced the flagellin protein and flagellin protein production was detectable even when the colonies were not lysed with chloroform. The detection of the flagellin protein with or without colony lysis suggested that the flagellin protein is expressed and transported to the cell surface. In addition, following enzymatic development with diaminobenzidene (diaminobenzidene is a precipitating substrate for the detection of peroxidase activity and produces a brown stain) *E. coli* cells containing pKW52 and pKW333 showed positive reactions almost as intense as

P. aeruginosa PA103 cells harboring those same plasmids. Whereas, *E. coli* cells containing pKW8 and pKW84 showed reactions less intense than *P. aeruginosa* PA103 cells harboring those plasmids. The difference in intensities of the colony blot reactions suggested that each of those clones contains different regions of the flagellin DNA which affected expression (Kelly-Wintenberg *et al.*, 1989).

Western blots of culture supernatants of the same transformed *E. coli* HB101 cells were separated in denaturing polyacrylamide gels. Each supernatant sample displayed a protein band of the approximate molecular weight of 53,000 and that same band reacted with the b-type polyclonal antiserum. The motility assays, colony and Western immunoblots showed that a DNA fragment containing the flagellin b gene had been cloned and expressed in *E. coli*. Limited endonuclease restriction maps of the clones suggest that pKW52 and pKW333 contain the same *P. aeruginosa* fla+ DNA. Also the limited mapping suggested that clones pKW84 and pKW102 contain overlapping, but different *P. aeruginosa* fla+ DNA (Kelly-Wintenberg *et al.*, 1989).

Statement of Purpose

Molecular biology and research applications have made a tremendous impact on diagnostic microbiology. Technological advances in molecular biology research applications has impacted hospital laboratories and clinics, industrial quality control laboratories, and other settings where diagnostic microbiology is routinely performed. The development of molecular biology techniques has made the detection and identification of microorganisms faster. The "conventional battery of biochemical tests performed in liquid media, followed by serotyping by use of polyclonal antisera derived from rabbits and carefully and laboriously absorbed with appropriate antigens to remove cross-reactive immunoglobulins" took days to weeks for definitive answers (Swaminathan *et al.*, 1989). Patient care and treatment may be improved with rapidly attained molecular diagnostic results guiding patient therapy.

Molecular biology techniques are important in the treatment of diseases that may cause morbidity and mortality in the immunocompromised, the very young or old, and those individuals with significant medical illness. For

example, pneumonia, bacteremia, and peritonitis infections, and intoxications such as tetanus and botulism require that detection and identification of the microorganisms that cause such diseases and product spoilage be done quickly for appropriate treatment or remedial measures. Rapid detection methods derived from molecular biology research applications have clinical significance. Some microorganisms (e.g *Mycobacterium*) are slow-growing or are difficult to culture. In addition, the use of molecular techniques may be cost and labor efficient. Conventional detection tests of toxins such as heat-labile and heat-stable toxins of *Escherichia coli* would be costly and laborious. On the other hand, molecular biology techniques are "specific, sensitive, and easy to perform" (Swaminathan *et al.*, 1989). This thesis research is an important component in the study of the *Pseudomonas aeruginosa* genomic library contained in the clone pKW52.

Major molecular biology techniques have been used to analyze pKW52. The b-type flagellin gene in clone pKW52 will be subcloned, sequenced, and characterized. Subcloning pKW52 is the first step in this thesis research. Clone pKW52 at approximately 49.1 kilobases long is a larger clone than is generally preferred to work with in molecular analysis. Based on the number of amino acids of *P. aeruginosa* strain M2 (b-type flagellin), the PAO1 flagellin b-type gene is estimated to be 1.7 kilobases in size. The next step in the research is to determine the nucleotide and amino acid sequence of the flagellin b-type gene. The nucleotide and amino acid sequence of the PAO1 flagellin b-type gene is necessary to examine the structure, investigate protein expression, and develop a complete restriction map of the gene. Furthermore, the homology and heterogeneity in flagellin structure among various bacteria will be investigated. The molecular analysis of *Pseudomonas aeruginosa* genomic library pLAFR1-PAO1 (clone pKW52) will provide additional methods of detection and identification of strain PAO1, and insights in the genetics of the flagellin b-type gene.

CHAPTER 2

MATERIALS AND METHODS

Bacteria and plasmids.

Pseudomonas aeruginosa strain PAO1 is a wild type strain of the species and is Fla+ (contains flagellin, the monomeric protein). *P. aeruginosa* strain PAO1 was supplied by A. Kropenski (Drake *et al.*, 1987). *P. aeruginosa* PA103 was used as a Fla- strain. PA103 cells do not synthesize a complete flagellar filament, although flagellin is detectable. Fla- mutants are not well characterized and are not readily isolated (Liu, 1966). *P. aeruginosa* strain PA103 is a laboratory strain. *P. aeruginosa* strains PAO1 and PA103 were used as controls in the colony immunoblotting experiments. In addition, *E. coli* (strain) HB101 cells and (strain) INVαF' cells with vector alone were used as controls in the colony immunoblotting experiments. The *E. coli* HB101 cells were supplied by Dr. Jeffery Becker (University of Tennessee, Knoxville, TN) and the *E. coli* INVαF' cells were obtained from Invitrogen Corporation (San Diego, CA).

Fragments of *Pseudomonas aeruginosa* strain PAO1 genome were subcloned in cosmid pLAFR1 (Kelly-Wintenberg *et al.*, 1989). Cosmid pLAFR1 is 21.6 kb, contains a single *EcoRI* site, is tetracycline resistant, and capable of replicating in a broad range of Gram negative bacteria (Ditta *et al.*, 1980). The cosmid construct containing the flagellin b-type gene is clone pKW52. *E. coli* HB101 cells harboring pKW52 were supplied by Dr. Kimberly-Kelly Wintenberg (University of Tennessee, Knoxville, TN).

The flagellin a-type gene of *Pseudomonas aeruginosa* strain PAK was cloned in pUC18. Plasmid pUC18 confers ampicillin resistance, contains a polylinker inserted within the alpha region of the lacZ gene, and can be amplified with chloramphenicol (Ausubel *et al.*, 1989). The cloned a-type flagellin was sequenced, and expressed in *E. coli* DH5α cells (Totten *et al.*, 1990). The plasmid construct containing the sequenced a-type flagellin gene is clone

pPT218. *E. coli* DH5α cells harboring clone pPT218 were supplied by Dr. Stephen Lory (University of Washington, Seattle, WA).

The plasmid construct containing the PCR amplified fragment of pKW52 is clone pKWII. *E. coli* strain INVαF' harbors clone pKWII. The cloning vector pCRII contains the *lacZα* complementation fragment for blue-white color screening, confers ampicillin and kanamycin resistance, and has a versatile polylinker (Mead, 1991).

Culture Conditions.

Bacteria were cultured on Luria-Bertani (LB) medium (Difco, Detroit, MI) containing 10g Bacto tryptone, 5g Bacto yeast extract, and 10g sodium chloride per liter. Bacteria for colony blots were prepared on Luria agar. Luria agar is LB medium and 15g/L of Bacto-agar (Maniatis *et al.*, 1989). For selection of clone pKW52 bacteria were cultured in LB medium containing 40μg/ml of tetracycline. For selection of clones pPT218 and pKWII bacteria were cultured in LB medium containing 50μg/ml of ampicillin. Cells for transformation experiments were cultured on Luria agar containing 50μg/ml of ampicillin and 25μl of X-Gal (40mg/ml stock in dimethylformamide).

Isolation of plasmid DNA.

Plasmid DNA was extracted for routine analysis using the lysis by alkali method (Ausubel *et al.*, 1989). Bacteria were grown in LB medium overnight at 37°C with the appropriate antibiotic. The different strains of bacteria were inoculated from -80°C stock cultures into 20ml of LB. After growth, these cells were inoculated into 1 liter of fresh LB with antibiotic and incubated at 37°C for 18-22 hours on a gyratory shaker. The cells were harvested by centrifugation for 30 minutes at 7000 x g. The cell pellets were retained following centrifugation and suspended in 10 ml of Solution I (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH8.0). Freshly prepared lysozyme solution (10mg/ml in 10mM Tris-HCl pH 8.0) was added to the cells. The cell suspension was mixed

thoroughly and incubated for 10 minutes at room temperature. Following incubation the cells were gently mixed with 20ml of freshly prepared Solution II (0.2N NaOH [freshly diluted from a 10N stock], 1% SDS). After a 10 minute incubation at room temperature, 15 ml of ice-cold Solution III (60ml 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml of water) was added to the cell suspension. The mixture was incubated for 10 minutes on ice followed by centrifugation for 30 minutes at 7000 x g. The supernatant was filtered through several layers of cheesecloth and the DNA precipitated with 0.6 volume of 100% ethanol and incubated overnight at -20°C. The DNA was pelleted by centrifugation for 30 minutes at 7000 x g, suspended in 1X TE (10mM Tris-HCL, 1mM EDTA pH 8.0), and then was used for routine analysis.

For purer preparations plasmid DNA was extracted using the Qiagen plasmid kit (Qiagen, Chatsworth, CA). The Qiagen plasmid purification protocol is based on the lysis by alkali procedure. Cells were grown in LB medium for 18-22 hours at 37°C, and were then harvested by centrifugation and suspended in 10 ml of solution P1 (100µg/ml RNase A, 50mM Tris-HCL, 10mM EDTA pH 8.0). The cells were gently mixed with 50 ml of solution P2 (200mM NaOH, 1% SDS). After a 5 minute incubation at room temperature, 50 ml of chilled solution P3 (3.0M KAc pH 5.5) was added to the cells. The mixture was incubated for 30 minutes on ice. The mixture was centrifuged twice 15 minutes each at 8000 x g and filtered through several layers of cheesecloth.

The supernatant was loaded onto a pre-equilibrated Qiagen-tip. The Qiagen-tip was washed with solution QC (1.0M NaCl, 50mM MOPS, 15% ethanol, pH 8.5). The DNA was eluted from the Qiagen-tip with 35ml of solution QF (1.25M NaCl, 50mM Tris-HCL, 15% ethanol, pH 8.5). The eluted plasmid DNA was precipitated with 0.7 volume of 100% ethanol and incubated overnight at -20°C. The DNA was pelleted for 20 minutes at 8000 x g. After centrifugation the pellet was washed with cold 70% ethanol and briefly air-dried. The purified plasmid DNA was resuspended in 1X TE pH 8.0 and used in sequencing, radiolabeling, and cloning procedures.

DNA procedures.

Restriction endonucleases were purchased from Gibco BRL (Gibco BRL, Grand Island, NY). Restriction endonuclease cleavage was performed by incubating the enzyme(s) with the DNA in appropriate reaction conditions. The amount of enzyme(s) and DNA, the buffer concentrations, and the duration of the reaction varied (Ausubel *et al.*, 1989).

Agarose gel electrophoresis was used to separate, identify, and purify plasmids, restriction fragments, and genomic DNA. The gels were prepared with 0.8% agarose (Sigma, St. Louis, MO) dissolved in 1X Tris-borate (5X: 54g Tris base, 27.5g boric acid, 20ml 0.5M EDTA pH 8.0) electrophoresis buffer. The agarose was melted in a microwave oven and poured between 0.5 and 1.0cm thick into a sealed gel casting platform. After the gel was solidified, the gel comb was removed and electrophoresis buffer was added to cover the gel just until the wells were submerged (Ausubel *et al.*, 1989).

The DNA samples were mixed with 6X sample buffer (0.25% bromophenol blue, 40% [w/v] sucrose in water) and loaded into the wells with a pipettor (Maniatis *et al.*, 1989). The voltage was set to a desired level and the gels were run until sufficient separation of the DNA fragments or the marker dye was near the end of the gel. The power was turned off, and the gel was removed and stained with ethidium bromide (50µl of 10mg/ml stock in 300ml of distilled water) (Sigma, St. Louis, MO) for 10 minutes with gentle shaking. The gels were destained by shaking in water for an additional 30 minutes. The stained DNA bands were viewed by illumination with UV light. The gel was photographed with a Polaroid camera (red-orange filter).

Lambda *HindIII* and 1kb molecular weight markers were generally loaded into wells on both the right and left sides of the agarose gel. The sizes of the DNA fragments were estimated by comparison of migration patterns to the marker DNAs of known size. The marker DNAs were purchased from commerical sources (Gibco BRL, Gaithersburg, MD).

DNA bands were electroeluted from agarose gels using NA 45 DEAE membranes (Scheichler & Schuell, Keene, NH) (Lizardi, 1984). The membranes were washed for 10 minutes with 10mM EDTA pH 7.6 and for 5 minutes with 0.5M NaOH followed by several rapid rinses in distilled water. After

electrophoretic separation of the DNA, a wet strip of membrane was placed in an incision just ahead of the band of interest. Electrophoresis was continued (at the same voltage or at an increased voltage) until the DNA bound to the membrane. Binding was determined by ethidium bromide fluorescence using long wave UV. The membrane was placed in a 0.5ml microcentrifuge tube and 150-250µl of NET buffer (0.15M NaCl, 0.1mM EDTA, 20 mM Tris-HCl pH 8.0) was added. The microcentrifuge tube was spun 5 seconds to completely submerge the strip. The DNA was eluted with incubation for 10-45 minutes at 55-68°C. Residual ethidium bromide was extracted from the eluted sample with 3 volumes of water-saturated n-butanol. The DNA was precipitated for 5 hours at -20°C with 2.5 volumes of ethanol. The DNA was reprecipitated with 0.3M sodium acetate and 2.5 volumes of ethanol.

Southern blotting.

Localization in the cloned DNAs of similar sequences to the radiolabeled probes was accomplished by Southern hybridization. Following agarose gel electrophoresis the DNA was nicked for 30 seconds with UV light (short wave), and transferred by vacuum blotting onto nylon membrane (ICN, Irvine, CA). DNAs were blotted for 50 minutes in a 0.4N NaOH solution (60 cm of pressure). The position of the gel wells was drawn on the membrane with a #2 pencil. The DNA was fixed to the nylon membrane by exposing the side of the membrane carrying the DNA to UV light for 30 seconds (short wave). The membrane was rinsed in 2X SSC solution (20X: 175.3g NaCl, 88.2g $Na_2C_6H_5O_7$ pH 7.0) or 2X SSPE solution (20X: 210g NaCl, 27.6g NaH_2PO_4, 7.4g EDTA pH 7.4) for 20 minutes at room temperature. The membrane was placed on Whatman 3MM filter paper to remove excess SSC or SSPE and then sealed in a bag. The volume of prehybridization solution added to the pouch was 0.2ml of solution for each square centimeter of membrane.

For high stringency conditions the prehybridization solution consisted of 50% formamide solution (100% stock concentration), 5X SSPE solution (20X stock concentration), 5X Denhardt's reagent (50X stock concentration), 0.3% SDS detergent (10% stock concentration), and water. For low stringency conditions

the prehybridization solution consisted of a 40% formamide solution (100% stock concentration), 5X SSPE solution (20X stock concentration), 5X Denhardt's reagent (20X stock concentration), 0.3% SDS detergent (1% stock concentration), and water. The membrane was incubated in the prehybridization solution for 2-4 hours at the appropriate temperature (37°C for low stringency conditions and 42°C for high stringency conditions).

The double-stranded radiolabeled probe was denatured by heating for 5 minutes in boiling water followed by rapidly chilling in ice water. After incubation the prehybridization solution was decanted from the pouch and the denatured probe and hybridization solutions were added. The recipe for the hybridization solution was the same as the prehybridization solution. The bag was incubated overnight at 37°C or 42°C. Wearing gloves the bag was removed from the incubator and a corner immediately cut off. The radioactive solution was poured out into a radioactive waste disposal container, and then the pouch was cut along the length of three sides to remove the membrane. The membrane was immediately washed to remove nonspecific radioactivity.

For low stringency conditions, the membrane was washed ten minutes twice at room temperature in 2X SSPE, 0.1% SDS. For high stringency conditions, the membrane was washed for five minutes at room temperature in 2X SSPE, 0.5% SDS; 15 minutes at room temperature in 2X SSPE, 0.1% SDS; and for 30 minutes at 37°C in 0.1X SSPE, 0.5% SDS. After completion of the washing steps, the membrane was placed on a sheet of Whatman 3MM filter paper to remove excess liquid. The damp membrane was wrapped in plastic wrap and exposed to Kodak XRP-1 X-Ray film at -70°C for 18-24 hours with an intensifying screen.

Radiolabeling probes.

Three radioactive DNA probes were used in the isolation and analysis of similar sequences in the cloned DNAs. An *EcoRI-HindIII* restriction digest of clone pPT218 yielded a 1.7kb fragment. The 1.7kb fragment is the cloned flagellin a-type gene. The 1.7kb fragment was electroeluted from agarose gels using NA 45 DEAE membranes in the exact manner described earlier. Following electroelution the DNA fragment was radiolabeled by random primer extension.

The purified DNA (50-250ng) was mixed with 2.5µl 10X Klenow buffer, 2.0µl dATP, 2.0µl GTP, 2.0µl TTP, 0.5µl primer, 2.5µl ^{32}P-dCTP (10µCi/µl), and 1µl Klenow (6U/µl). The labeling reaction was incubated for 60 minutes at room temperature.

The second probe designated 1N is an oligonucleotide of the N-terminal nucleotide sequence 5'- GAA CGC CAA CGA CGG of the PAK flagellin a-type gene. Probe 1N was endlabeled with [γ-^{32}P] dATP using polynucleotide kinase (ICN, Costa Mesa, CA) (Maniatis *et al.*, 1989). The labeling reaction consisted of 2µl oligonucleotide 1N (10µM), 13µl of water, 3µl of 10X kinase buffer, 10µl [γ-^{32}P] dATP, and 2µl polynucleotide kinase (10U/µl). Following a 30 minute incubation at 37°C the reaction was stopped with the addition of 70µl of 1X TE pH 8.0.

The 600bp fragment resulting from the PCR amplification of clone pKW52 was the third probe used in Southern hybridization experiments. The 600bp fragment was electroeluted from agarose gels using NA 45 DEAE membranes in the exact manner described earlier. Following electroelution the DNA fragment (50-250ng) was radiolabeled by random primer extension in the exact manner described earlier.

Following labeling the probes were purified with BioSpin-6 columns. Each column contains Bio-Gel P-6 polyacrylamide gel in 0.8 ml of SSC pH 7.0 (0.15M NaCl, 17.5mM $Na_2C_6H_5O_7$) with 0.02% sodium azide. The gel matrix was resuspended, and the buffer drained and discarded. The BioSpin-6 column was placed in a collection tube and centrifuged for 2 minutes at approximately 1100 x g to remove the remaining liquid from the column. After centrifugation the collected buffer and collection tube were discarded. The radioactive probe was applied directly to the center of the column with a micropipetter. The column was centrifuged 1100 x g for 4 minutes at room temperature. The purified probe was collected in the collection tube and added to the hybridization solution.

Attempted isolation of flagellin b-type gene in clone pKW52 by PCR.

Polymerase chain reaction (PCR) was used to amplify DNA sequences in clones pKW52 and pPT218. Oligonucleotides designated 1N and 1C (Integrated DNA Technologies, Coralville, IA) were the primers used in the PCR experiments. Oligonucleotides 1N and 1C are 15-mers derived from the nucleotide sequence of the PAK flagellin a-type gene. Oligonucleotide 1N sequences are located within a twelve N-terminal amino acid sequence (ALTVNTNASLNT) identical in the PAK flagellin a-type gene and the PAO1 flagellin b-type gene (Anderson *et al.*, unpublished report). The sequence of 1N is 5' - GAA CGC CAA CGA CGG (Totten *et al.*, 1990). The flagellin C-terminal amino acid sequences are conserved in bacteria (Totten *et al.*, 1990). Oligonucleotide 1C sequences are located within the C-terminal of the PAK flagellin a-type gene. The sequence of 1C is 5' - GCG ACC GAG GTC GGC (Totten *et al.*, 1990). Clone pKW52 and pPT218 were extracted with the Qiagen kit in the exact manner described previously. The GeneAmp® PCR Reagent Kit (Roche Molecular Systems, Inc., Branchburg, NJ) with AmpliTaq® DNA Polymerase was used for PCR experiments. The reaction mixture consisted of 39.5µl sterile, deionized, distilled water; 5µl 10X PCR Buffer II; 1µl dATP, 1µl dGTP, 1µl dTTP, 1µl dCTP; 0.5µl AmpliTaq® Polymerase; 5µl Primer 1N (1:10 dilution of 200 picomoles/µl stock); 5µl 1C (1:10 dilution of 200 picomoles/µl stock); and 1µl 10 ng/µl template. Once assembled, the mixture was overlaid with 2 drops of mineral oil (Sigma, St. Louis, MO) and cycled 38 times. Each cycle consisted of denaturation for 1 minute at 94°C, annealing for 1 minute at 42°C, and extension for 2 minutes at 72°C. PCR experimental conditions were identical for the amplification of homologous sequences in clone pPT218 (flagellin a-type). The PCR products were then displayed on an agarose gel and examined for specificity.

Construction of clone pKWII.

The PCR amplified fragment of pKW52 was electroeluted from an agarose gel in the exact manner described earlier and ligated into the *EcoRI* sites

of vector pCRII using the TA Cloning® kit. The ligation reaction consisted of 5µl sterile water, 1µl 10X Ligation Buffer, 2µl pCRII vector (25 ng/µl), 1µl PCR product, and 1µl T4 DNA Ligase. The amount of PCR product ligated was determined by the equation Xng PCR product = (Y bp PCR product) x (50 ng pCRII vector)/ (size in bp of the pCRII vector). X ng is the amount of PCR product of Y base pairs to be ligated for a 1:1 molar ratio. The ligation reaction mixture was incubated overnight at 16°C overnight. The ligated PCR product-pCRII vector (1ml) was transformed into TA Cloning® One Shot competent cells. The transformation reaction was mixed with 2µl of beta-mercaptoethanol followed by incubation for 30 minutes on ice. After incubation the transformation reaction was placed for 30 seconds in a 42°C water bath and then on ice for 2 minutes. The cells were incubated for one hour at 37°C in SOC medium. The vial of transformed cells was placed on ice and 25µl and 100µl aliquots were spread onto freshly prepared Luria agar plates. The Luria agar plates contained 50µg/ml of ampicillin. Using a glass spreader, 25µl of X-Gal (40mg/ml in dimethylformamide) was spreaded on top of the agar and allowed to diffuse into the medium for one hour. The inoculated plates were incubated overnight at 37°C. White colonies were picked for plasmid isolation and restriction analysis.

Colony immunoblotting.

Colony immunoblotting with monoclonal antibodies was used to confirm the antigenicity of the clone DNAs. The colony blot procedure used is a similar procedure used by Nicas (Nicas *et al.*, 1985). *E. coli* HB101 cells harboring pKW52, *E. coli* cells harboring pKWII, *E. coli* HB101 cells, *E. coli* INVαF' cells, PAO1 cells, and PA103 cells were plated onto Luria agar with antibiotic, and then were grown overnight at 37°C. The colonies were lifted off the medium with dry nitrocellulose filters previously washed in a 0.2% SDS PBS solution(1.93g/L NaH_2PO_4-H_2O, 9.66 g/L Na_2HPO_4-H_2O, 1.63g/L NaCl). Colonies were lysed in a chloroform saturated chamber, and were then blocked in a 3% gelatin-PBS solution. After washing in PBS the filters were incubated for two hours at 37°C in human anti-a monoclonal (1:5000 dilution) (5mg/ml), human anti-b-type

monoclonal (1:5000) (5mg/ml), anti-1210 (a-type) polyclonal (1:5000), and anti-M2 (b-type) polyclonal (1:1000). The filters were washed with PBS and incubated for 1 hour 30 minutes at 37°C in a 1:10000 dilution of horseradish peroxidase conjugated goat anti-human or goat anti-rabbit IgG antibody (Bio-Rad, Hercules, CA). The filters were rinsed with wash buffer (10mM NaCl, 5mM EDTA, 200mM Tris base pH 7.4, 5ml per liter Triton X-100) and deionized water before enzymatic development with 3,3'-diaminobenzidine tetrahydrochloride (DAB) tablet sets (Sigma, St. Louis, MO).

Each tablet set contains DAB (0.7mg/ml), urea hydrogen peroxide (0.7mg/ml), and Tris buffer (0.06M) when dissolved in 15ml of water. The required number of DAB and urea hydrogen peroxide tablets were dissolved in the appropriate volume of deionized water. The colony blots were immediately submerged in the DAB substrate for a maximum of 5 minutes in the absence of light. The reaction was stopped by gently rinsing the filters with deionized water. Both human anti-a and human anti-b monoclonal antibodies were prepared by Baxter Healthcare Corporation (Baxter Healthcare Corporation Hyland Division, Duarte, CA). The anti-1210 polyclonal and the anti-M2 polyclonal antisera were prepared by Dr. Thomas Montie's laboratory (University of Tennessee, Knoxville, TN).

DNA Sequencing.

Clone pKWII was sequenced using the Sequenase® Version 2.0 DNA Sequencing Kit and an automated sequencer. The sequencing procedure using the kit consisted of denaturation, annealing, labeling, and termination steps. Qiagen extracted double stranded DNA (3-5µg) was alkaline-denatured with 0.1 volume of 2M NaOH, 2mM EDTA and incubated for 30 minutes at 37°C. The denatured DNA was neutralized with 0.1 volume of 3M sodium acetate (pH 4.5-5.5) and precipitated with 2-4 volumes of ethanol for 15 minutes at -70°C. The precipitated DNA was washed with 70% ethanol and dissolved in 7µl of distilled water, 2µl of Sequenase reaction buffer, and 1µl of primer. Universal M13 Forward (-20) (5' -GTA AAA CGA CGG CCA G) and Universal M13 Reverse (5' -CAG GAA ACA GCT ATG AC) (Invitrogen, San Diego, CA) were primers used

in generating the nucleotide sequences of the insert of pKWII. The universal primers are complementary to pCRII.

The denatured DNA was heated at 65°C for 2 minutes. The heated sample was set on a benchtop and allowed to cool slowly to <35°C. After cooling the sample was centrifuged briefly and chilled on ice. The labeling reagents were added to the chilled reaction. The labeling reagents were 1µl DTT (0.1M), 0.5µl [α-^{35}S] dATP (10µl Ci/ml), and 2µl diluted Sequenase Version 2.0 enzyme (diluted 1:8 in dilution buffer). Aliquots of 3.5µl of labeling reaction were added to 2.5µl ddGTP, 2.5µl ddATP, 2.5µl ddTTP, or 2.5µl ddCTP. The tubes containing the termination mixes were prewarmed for 1 minute at 37°C before the additon of the labeling reaction. The termination reaction was incubated for 5-30 minutes at 37°C. The incubation ended with the addition of 4µl of stop solution (95% Formamide, 20mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF) to the termination reactions. The reactions were mixed thoroughly and stored on ice until ready to load the sequencing gel.

Clone pKWII was primer extension sequenced by Ric Sugarek (Lark Sequencing Technologies, Inc., Houston, TX) using primers Universal M13 Forward (⁻20) and Universal M13 Reverse. An electropherogram was the sequencing report of the insert of pKWII generated by Applied Biosystems Data Analysis software. The electropherogram represented the raw data of the sequencing gel. The sequences were printed on a Macintosh computer-compatible diskette using Sequencher™ 2.1 software. The sequences generated by primer extension of the pKWII insert were investigated using Genetics Computer Group (GCG) (Madison, WI) and BLAST search programs (Netscape, Internet).

Sequencing Gel Preparation and Running.

The Sequi-Gen® Nucleic Acid Sequencing Cell (Bio-Rad, Hercules, CA) was used for gel electrophoresis of reactions prepared by the Sequenase® Version 2.0 DNA Sequencing Kit. The Sequi-Gen apparatus is a vertical instrument with a cell the dimensions 38 x 50 cm. The actual gel area is 34 x 50 cm. The Sequi-Gen cell apparatus was set-up according to the manufacturer's

instructions. The gel solution (150ml) was prepared with 63g urea, 15ml of 10X TBE, and 25ml of 30% acrylamide stock solution (28.5g acrylamide and 1.5g bis-acrylamide dissolved up to 100ml with distilled water). The gel solution was filtered through a 0.45 micron mesh filter and degassed under strong vacuum for 20 minutes. The gel solution was solidified with the addition of 1µl of 25% ammonium persulfate solution and 1µl of TEMED per ml of gel solution. The gel was casted according to the manufacturer's instructions.

The gels were pre-run for 15-60 minutes at 50-55°C. The iced samples were heated for 3 minutes at 95°C, quenched-cooled, and loaded immediately into the rinsed wells. The gel was electrophoresed at a constant voltage of 1800-2000V until the marker dye was near the end of the gel. The power was turned off, and the gel was removed and soaked in a 10% acetic acid, 10% methanol solution for 15-20 minutes at room temperature. Excess acetic acid and methanol were removed from the gel surface with paper towels. The sequencing gel was transferred to Whatman 3MM filter paper and covered with plastic wrap. The edges of the gel were trimmed several inches along the sides and dried for 30 minutes at 80°C with a gel dryer. The gel was exposed to Kodak XAR-5 X-Ray film for 24-48 hours at -80°C. An intensifying screen was not used.

Computer-assisted Analysis.

The computer-assisted investigation of the sequences of the insert of pKWII was performed with GCG sequence analysis (Madison, WI) and the BLAST nucleotide search programs. The investigation of the sequences of the insert of pKWII consisted: 1) identification of the primer sequences in the insert, and 2) the determination of the sequence identity between the insert and the PAK flagellin a-type gene. The investigation of the insert sequences was begun using GCG's fastA program. FastA is a method developed by Pearson and Lipman that searches for similiarities between one sequence (query) and any group of sequences. FastA is a multiple step search program in which the computer finds the perfect or "best" match between the comparison sequences (GCG Program Manual, 1994). In addition, using fastA the similarity or homology of the perfect or "best" match between the comparison sequences was described by a

percentage of identity (GCG Program Manual, 1994). Computer symbols for identity were a vertical pair of dots (:) and a vertical solid line to note similarity and homology, respectively.

The translation of the nucleotide sequences of the insert of pKWII in six possible reading frames was determined using tfastA. TfastA is a method developed by Pearson and Lipman that searches for similarities between a query peptide sequence and any group of nucleotide sequences (GCG Program Manual, 1994). The nucleotide sequences were translated in six frames before the comparison. The BLAST nucleotide search program of the National Center for Biotechnology Information (NCBI) was used to identify nucleotide sequences in the NCBI database that have identity to the sequences of the insert of pKWII. The BLAST method is a Netscape computer program. Netscape is located on the Internet. BLAST searches were faster, and more specific in identifying those sequences with similarity to the target sequences than searches using GCG. In addition, the use of the BLAST search program was not charged to the user's VAX account.

CHAPTER 3

RESULTS

Attempts to identify the cloned flagellin b-type gene of pKW52 by PCR amplification.

PCR was an attempt to locate the flagellin b-type gene in clone pKW52 previously isolated by Wintenberg *et al.*, 1989. PCR was used to locate this gene, because it is a rapid procedure to amplify DNA sequences. Primers designated 1N and 1C **(Figures 1, 2)** were synthesized, and then used to amplify the flagellin b-type gene in clone pKW52. Primers 1N and 1C were derived from the N- and C- terminal nucleotide sequences of the PAK flagellin a-type gene. Primers 1N and 1C were selected for amplification of the flagellin b-type gene, because of their expected specificity for the flagellin b-type gene. Anderson *et al.*, 1989 reported that a portion of the N-terminal amino acid sequence is identical in the PAK flagellin a-type gene and PAO1 flagellin b-type gene. It was subsequently confirmed that an N-terminal 12 amino acid sequence was identical in the PAK flagellin a-type gene and PAO1 flagellin b-type gene. The 15 base nucleotide sequence of primer 1N was located in the 12 amino acid sequence ALTVNTNASLNT. Although it had not been reported that C-terminal sequences were identical in the PAK flagellin a-type gene and PAO1 flagellin b-type gene, Totten *et al* 1990 reported the C-terminal amino acid sequences of bacterial flagellins are highly conserved. The 15 base nucleotide sequence of primer 1C was selected from the C-terminal sequences of the PAK flagellin a-type gene. No other amino acid sequence data was available for *P. aeruginosa* flagellin b-type strains M2 and PJ108. Degenerate primers were not used to amplify the flagellin b-type gene in clone pKW52, because it was hypothesized that primers 1N and 1C would be complementary to the target sequences, and would amplify a specific product.

1 GAACGCCAAC GACGG

Figure 1. The 15 base (5' - 3') sequence of oligonucleotide 1N. Oligonucleotide 1N is derived from the nucleotide sequence of the *P. aeruginosa* PAK flagellin a-type gene. Primer 1N was used to amplify the flagellin b-type gene in clone pKW52.

1 GCGACCGAGG TCGGC

Figure 2. The 15 base (5' - 3') sequence of oligonucleotide 1C. Oligonucleotide 1C is derived from the nucleotide sequence of the *P. aeruginosa* PAK flagellin a-type gene. Primer 1C was used to amplify the flagellin b-type gene in clone pKW52.

E. coli HB101 cells harboring pKW52 and *E. coli* DH5α cells harboring pPT218 (flagellin a-type gene) were each grown overnight. The DNA was extracted using the Qiagen kit, and 10ng/μl of pure plasmid DNA was amplified 38 cycles. Since primers 1N and 1C were derived from the nucleotide sequence of the PAK flagellin a-type gene, it was expected that the primers would anneal and amplify the flagellin a-type gene of pPT218.

The amplification of pKW52 with primers 1N and 1C yielded three bands observed with agarose gel electrophoresis and ethidium bromide fluorescence. Agarose gel electrophoresis of the PCR products is shown in **Figure 3**. To estimate the size of the fragments resulting from PCR amplification of clone pKW52, the size of the fragment resulting from PCR amplification of clone pPT218 (flagellin a-type gene) with primers 1N and 1C was determined. The sequences of primers 1N and 1C were identified in the sequence of the PAK flagellin a-type gene. Primer 1N sequences were identified at bases 605-619 **(Figure 4)**. Primer 1C sequences were identified at bases 1373-1387 **(Figure 5)**. Based on the location of primer 1N and 1C in the sequences of the PAK flagellin a-type gene, primers 1N and 1C would amplify a 768bp fragment in *E. coli* clone pPT218 from Lory. Comparison of the migration of the PCR amplified fragment of clone pPT218 (768bp in size) and molecular weight markers (Lambda *HindIII* and 1kb) with the PCR amplified fragment of pKW52, it was estimated the amplified fragment of pKW52 was 600bp in size **(Figure 3, lane 5)**.

The size of the first band **(Figure 3, lane 5)** was approximately 1.3kb. The size of the second band **(Figure 3, lane 5)** was approximately 600bp. The third band **(Figure 3, lane 5)** was less than 507bp in size. The third band was excess primer. The amplification of pKW52 with primers 1N and 1C yielded three bands, because primers 1N and 1C were not specific for pKW52. A technique to identify nonspecific PCR products is discussed in **Chapter 4 (Discussion)**. The amplification of pPT218 yielded two bands as shown in **Figure 3**. The first band **(Figure 3, lane 4)** was approximately 1.0kb. The second band **(Figure 3, lane 4)** was less than 507bp in size. The second band was excess primer **(refer to Figure 3, lane 5)**.

Figure 3. Agarose gel electrophoresis of the PCR amplification products of clones pKW52 and pPT218. Lane (1) 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(2)** PCR amplification products of pKW52 (1µl diluted 1:1000; 5µl of primers 1N and 1C diluted 1:2), **(3)** PCR amplification pKW52 (1µl diluted 1:1000; 5µl of primers 1N and 1C diluted 1:10), **(4)** PCR amplification pPT218 (1µl diluted 1:1000; 5µl of primers 1N and 1C diluted 1:2), **(5)** PCR amplification pKW52 (1µl diluted 1:100; 5µl of primers 1N and 1C diluted 1:2), **(6)** PCR amplification pKW52 (1µl diluted 1:10; 5µl of primers 1N and 1C diluted 1:2), **(7)** 1 µl 1kb Molecular Weight Marker (1:20 dilution of stock [250µg/242.7µl]), **(8)** No sample loaded in the well. **The concentration of the primers (1N, 1C) is 200 picomoles/µl.**

```
100.0% identity in 15 bp overlap

                                        10
                                GAACGCCAACGACGG
                                |||||||||||||||
CCAGGTCAACGGCCTGAACGTGGCTACCAAGAACGCCAACGACGGTATCTCCCTGGCGCA
   580       590       600       610       620       630

GACCGCTGAAGGCGCCCTGCAGCAGTCGACCAACATCCTGCAGCGTATGCGTGACCTGTC
   640       650       660       670       680       690
```

Figure 4. Location of primer 1N sequences in the PAK flagellin a-type gene. Primer 1N is a 15bp oligonucleotide derived from the nucleotide sequence of the PAK flagellin a-type gene. Primer 1N sequences are located at bases 605-619 of the PAK flagellin a-type gene.

```
100.0% identity in 15 bp overlap

                                        9
                                GCCGACCTCGGTCGC
                                |||||||||||||||
GACGGCGATCAAGCAGATCGACGCCAGCGTGCCGACCTCGGTCGCGGTGCAGAACCGCTT
    1350      1360      1370      1380      1390      1400

CGACAACACCATCAACAACCTGAAGAACATCGGTGAGAACGTATCGGCTGCTCGCGGCCG
    1410      1420      1430      1440      1450      1460
```

Figure 5. Location of primer 1C sequences in the PAK flagellin a-type gene. Primer 1C is a 15bp oligonucleotide derived from the nucleotide sequence of the PAK flagellin a-type gene. Primer 1C sequences are located at bases 1373-1387 of the PAK flagellin a-type gene.

Subcloning of the PCR-amplified fragment of pKW52.

Due to its approximation in size compared to the 768bp PCR amplified fragment of clone pPT218 (used as a control), it was hypothesized that the 600bp fragment of clone pKW52 may be the amplified flagellin b-type gene. The 600bp fragment resulting from the PCR amplification of pKW52 was electroeluted from an agarose gel, precipitated, and cloned into vector pCRII **(Figure 3, lane 5)**. The 600bp fragment was cloned using the TA Cloning® kit. The TA Cloning® kit offers several advantages over traditional cloning strategies, because modifying enzymes, purification, or restriction digestion is not required for ligation of the PCR-amplified insert to the vector. The presence of three bands in the PCR product of clone pKW52 made it necessary to electroelute the 600bp fragment from an agarose gel before ligation **(Figure 3)**. The ligation reaction of a 1:1 ratio of pCRII:600bp fragment was incubated overnight at 16°C. With ligation of the 600bp fragment of pKW52 to pCRII, the 600bp fragment was inserted into the *EcoRI* sites of the cloning vector disrupting the *lac*Zα gene. *E. coli* INVαF' competent cells were transformed with the ligation mixture pCRII:600bp fragment, and the transformed cells were then inoculated onto Luria agar plates containing ampicillin and X-Gal. The plates were incubated overnight at 37°C, and blue and white colonies resulted. The TA Cloning® kit was useful, because the blue and white colonies provide a screening procedure to determine those cells that may contain the 600bp insert. The insertion of the 600bp fragment in pCRII disrupts the *lac*Zα gene usually producing white cells. The blue colonies do not harbor the insert. The white colonies resulting from the cloning experiment were inoculated onto LB plates containing ampicillin, each colony numbered, and the DNA extracted from the clones by alkaline lysis.

After extraction of the plasmid DNA, each clone was digested with the restriction endonuclease *EcoRI* to examine the restriction pattern of the clones. The insertion of the PCR product into the *EcoRI* sites of pCRII should result in two fragments with the digestion of pKWII with *EcoRI*. Therefore, it was expected with the cloning of the 600bp fragment into the *EcoRI* sites of pCRII that two fragments, 3.9kb (pCRII) and 600bp (PCR product) in size, would result **(Figure 6)**. Restriction digestion with *EcoRI* demonstrated that colony #2 later designated clone pKWII contained an insert **(Figure 7, lane 6)**. The insert was

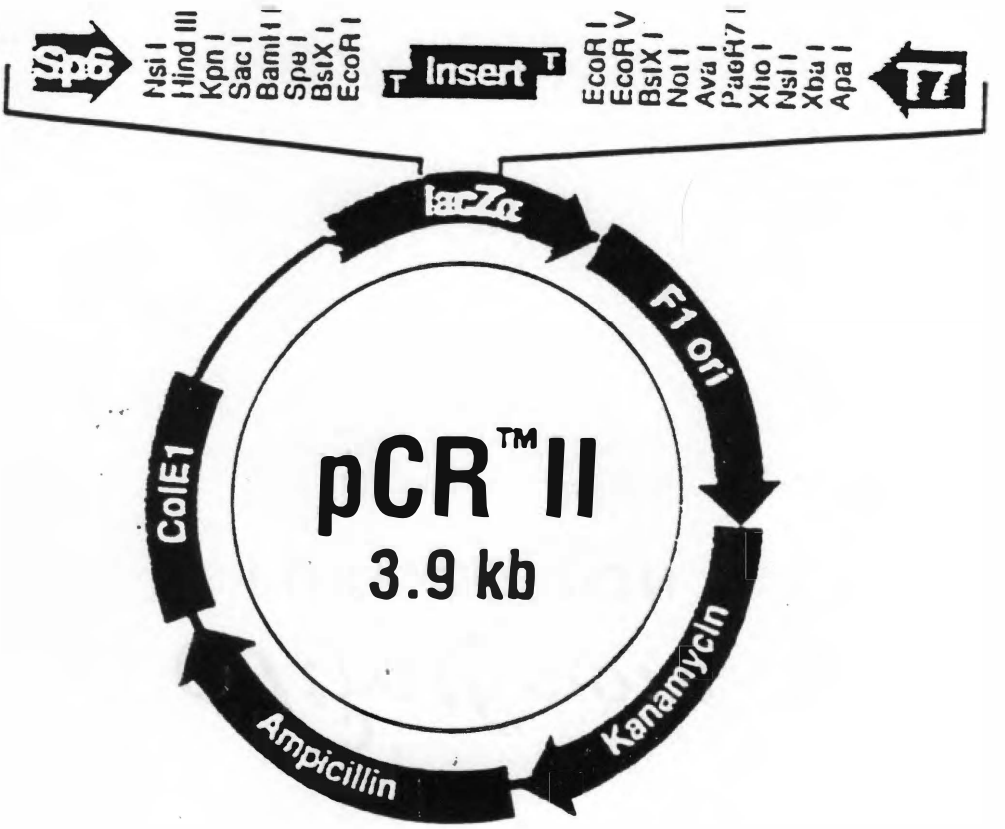

Figure 6. Diagram of the TA Cloning® vector pCRII. The vector pCRII contains the *lacZα* complementation fragment for blue white color screening, confers ampicillin and kanamycin resistance, and has a versatile polylinker. The pCRII vector has sixteen cloning sites in the polylinker for excision of inserts (Mead, 1991). The PCR amplified 600bp fragment of clone pKW52 was inserted in the *EcoRI* sites of the cloning vector.

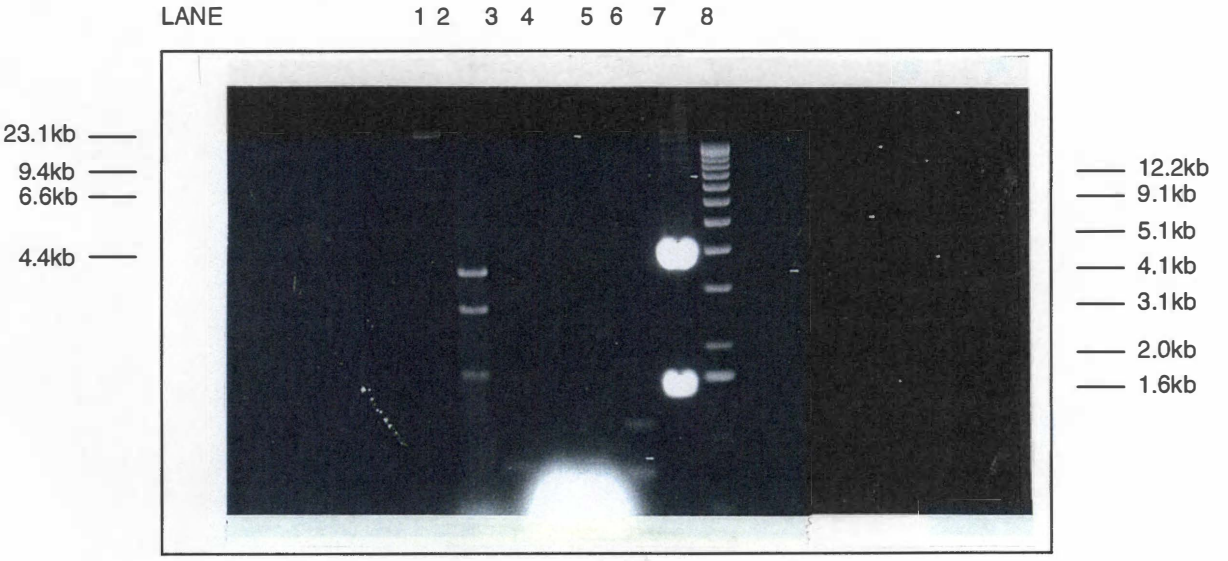

Figure 7. Agarose gel electrophoresis of restriction digests of clones pPT218, pKW52, and pKWII, and the PCR products of clones pPT218 and pKW52. **Lane (1)** 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(2)** Clone pPT218 plasmid DNA cut with *EcoRI-HindIII*, **(3)** PCR amplification products of clone pPT218, **(4)** Clone pKW52 plasmid DNA uncut, **(5)** Clone pKW52 cosmid DNA cut with *KpnI*, **(6)** PCR amplification products of clone pKW52, **(7)** Clone pKWII plasmid DNA cut with *EcoRI*, **(8)** 1 µl 1kb Molecular Weight Marker (1:20 dilution of stock [250µg/242.7µl]).

1.3kb in size, and the fragment was not the size expected with *EcoRI* digestion of pKWII **(Figure 7, lane 6)**. The 1.3kb insert is approximately twice the size of the 600bp PCR amplified fragment **(Figure 3, lane 5)**. The insert of pKWII was postulated to be two 600bp fragments that had ligated, and were then inserted in the *EcoRI* sites of pCRII. The indication that two 600bp fragments had ligated to form the approximately 1.3kb insert would be determined by sequence analysis of the insert of pKWII. In addition, the sequences of the insert of pKWII **(Figures 8, 9)** were investigated to determine identity with the PAK flagellin a-type gene sequences.

Sequence data from the pKWII insert.

The PCR amplified insert of clone pKWII was sequenced using the Sequenase® Version 2.0 DNA Sequencing Kit and an automated sequencer. Using the Sequenase® Version 2.0 DNA kit the pKWII insert was sequenced with primers Universal M13 Forward (-20), Universal M13 Reverse, and Reverse 1B (RV1B) to read 302 bases. Using an automated sequencer the Universal M13 Forward (-20) and Universal M13 Reverse primers read 802 bases. The sequence analysis of the insert of pKWII began with the identification of the location of primers 1N and 1C. FastA (computer program) investigation of primer 1N sequences and the sequences of the insert of pKWII generated by the Universal M13 Forward (-20) primer revealed identity (100.0%) in a 15bp overlap **(Figure 10)**. FastA investigation of primer 1N sequences and the sequences of the insert of pKWII generated by the Universal M13 Reverse primer revealed identity (100.0%) in a 15bp overlap **(Figure 11)**.

FastA investigation of primer 1C sequences and the sequences of the insert of pKWII generated by the Universal M13 Forward (-20) primer revealed no identity (80.0% in a 10bp overlap) **(Figure 12)**. FastA investigation of primer 1C sequences and the sequences of the insert of pKWII generated by the Universal M13 Reverse primer revealed no identity (76.9% in a 13bp overlap) **(Figure 13)**. Based on computer-assisted (fastA) analysis of the sequences of the insert of pKWII, it was determined that primer 1N annealed and amplified the insert of clone pKWII. In support of the fastA analysis, the computer alignment of the

```
  1  GGCTTGAACG CCAACGACGG TACGCTGTCA GTGATGGTGG CGGCGACAAG
 51  GCTATTTTCG ACAAATACTA TGATTTGATG AAAGCGATGG CGGGTTCCGT
101  GGTGCATACC GGGGAAATCG GTGCAGGTAA CGTCACCAAA CTGGCAAATC
151  AGGTCATTGT GGCGCTGAAT ATTGCCGCGA TGTCAGAAGC GTTAACGCTG
201  GCAACTAAAG CGGGCGTTAA CCCGGACCTG GTTTATCAGG CAATTCGCGG
251  TGGACTGGCG GGCAGTACCG TGCTGGATGC CAAAGCGCCG ATGGTGATGG
301  ACCGCAACTT CAAGCCGGGC TTCCGTATTG ATCTGCATAT TAAGGATCTG
351  GCGAATCGCG CTGGATACTT CTCACGGCGT CGGCGCACAA CTGCCGCTCA
401  CAGCTGCGG
```

Figure 8. Sequences of the insert of pKWII generated (automated sequencer) by Universal M13 Forward (-20) primer.

```
  1  GGCTTGAACG CCAACGACGG CAACCGGAAC AGAAACGTAC TGTGCATCAG
 51  GAAAAATTTC CCGAAATCCT TTTTCTATCG CCTGCGCAAC CTCGCTGGCA
101  GATAAACTTT CTTTATAAGA GTCTGGGGCG ATTACGATTT TCATACCTAT
151  GCCTGTTACC ACATGACGCC GGAGGGCGTT TCTCTTATTC GGCCTGGATT
201  CCAGGCCCGG ATTGCAATAC GCCATCCGGG CACGACGTCA TTAACGAGTA
251  ACTTCGACTT TCGCCAGTTT TTCGTAGTAG CACGCCAGGG CGCTATGATC
301  CGCCGTTCCT AAACCATCTG TTCGCAGTGC CTGCATCATC TCCATAACCG
351  CAGCTGTGAG CGGAAGTTGT GCGCCGACGC CGTGAGAAGT ATC
```

Figure 9. Sequences of the insert of pKWII generated (automated sequencer) by Universal M13 Reverse primer.

```
Sequences read by Universal M13 Forward (-20)

SCORES            Init1:    60 Initn:    60 Opt:    60
           100.0% identity in 15 bp overlap

                    10
             GAACGCCAACGACGG
             |||||||||||||||
       GGCTTGAACGCCAACGACGGTACGCTGTCAGTGATGGTGGCGGCGACAAGGCTATTTTCG
               10        20        30        40        50        60
```

Figure 10. FastA investigation of primer 1N and the sequences of the insert of pKWII generated by the Universal M13 Forward (-20) primer. Fasta investigation revealed identity (100.0%) in a 15bp overlap.

```
sequences read by Universal M13 Reverse

SCORES            Init1:    60 Initn:    60 Opt:    60
           100.0% identity in 15 bp overlap

                    10
             GAACGCCAACGACGG
             |||||||||||||||
       GGCTTGAACGCCAACGACGGCAACCGGAACAGAAACGTACTGTGCATCAGGAAAAATTTC
               10        20        30        40        50        60
```

Figure 11. FastA investigation of primer 1N and the sequences of the insert of pKWII generated by the Universal M13 Reverse primer. Fasta investigation revealed identity (100.0%) in a 15bp overlap.

```
SCORES
        80.0% identity in 10 bp overlap

                                        10
                                GCGACCGAGGTCGGC
                                     ||  ||||||
GATCTGGCGAATCGCGCTGGATACTTCTCACGGCGTCGGCGCACAACTGCCGCTCACAGC
   350       360       370       380       390       400

TGCGG
```

Figure 12. FastA investigation of primer 1C and the sequences of the insert of pKWII generated by the Universal M13 Forward (-20) primer. Fasta investigation revealed no identity (80.0% in a 10bp overlap).

```
SCORES
        76.9% identity in 13 bp overlap

                                        9
                                GCCGACCTCGGTCGC
                                  | |||||| | ||
TTTCCCGAAATCCTTTTTCTATCGCCTGCGCAACCTCGCTGGCAGATAAACTTTCTTTAT
  60        70        80        90       100       110

AAGAGTCTGGGGCGATTACGATTTTCATACCTATGCCTGTTACCACATGACGCCGGAGGG
 120       130       140       150       160       170
```

Figure 13. FastA investigation of primer 1C and the sequences of the insert of pKWII generated by the Universal M13 Reverse primer. Fasta investigation revealed no identity (76.9% in a 13bp overlap). The primer 1C sequences are in reverse orientation. Primer 1C sequences 5'- GCGACCGAGGTCGGC revealed 0.0% identity with the sequences of the insert of pKWII generated by the Universal M13 Reverse primer.

sequences generated by the Universal M13 Forward (-20) and Universal M13 Reverse primers included the 15bp (100.0%) sequence of oligonucleotide 1N **(Figure 14)**. In addition, primer 1C was shown by computer-assisted analysis not to have annealed and amplified the insert of clone pKWII.

Using tfastA (computer program) the nucleotide sequences of the insert of pKWII were translated into all six possible reading frames. In addition, the translated sequences were investigated to determine the amino acid identity between the insert and the PAK flagellin a-type gene **(Figure 15, 16)**. With tfastA investigation it was revealed that amino acid identity between the insert and the PAK flagellin a-type gene may be 50.0% in a 12aa overlap (frame 1), 66.7% in a 3aa overlap (frame 2), 19.6% in a 56aa overlap (frame 3), 33.3% in an 18aa overlap (frame 4), 21.4% in a 14aa overlap (frame 5), 13.2% in a 38aa overlap (frame 6) (Universal M13 Forward -20 sequences), 27.8% in an 18aa overlap (frame 1), 26.3% in a 38aa overlap (frame 2), 62.5% in an 8aa overlap (frame 3), 38.5% in a 13aa overlap (frame 4), 100.0% in a 3aa overlap (frame 5), or 36.4% in an 11aa overlap (frame 6) (Universal M13 Reverse sequences). The nucleotide sequences of the insert of pKWII were investigated to determine the sequence identity between the insert of pKWII and the PAK flagellin a-type gene. Fasta investigation of the insert of pKWII revealed identity (72.5%, 70.0% with respect to Universal M13 Forward and Universal M13 Reverse generated sequences) with the PAK flagellin a-type gene bases 934-972 **(Figures 17, 18)**.

Primer amplification and sequence identity with the PAK flagellin a-type gene demonstrated that clone pKWII does not contain the flagellin b-type gene. This conclusion was supported by BLAST (computer program) database searches. Using the BLAST nucleotide program, the database of NCBI was searched for sequences having identity with the insert of pKWII. The BLAST search program matched the nucleotide sequences of the insert of pKWII with *Escherichia coli* and *Haemophilus influenzae* sequences **(Figures 19, 20)**. As discussed in **Chapter 4 (Discussion)** these sequence matches with the insert of pKWII indicate that the flagellin b-type gene was not subcloned.

```
SCORES
        62.5% identity in 72 bp overlap

            10        20        30        40        50        60
GGCTTGAACGCCAACGACGGCAACCGGAACAGAAACGTACTGTGCATCAGGAAAAATTTC
||||||||||||||||||||| |  | |  |||  | |      ||  || |   | ||||
GGCTTGAACGCCAACGACGGTACGCTG-TCAGTGATGGTGGCGGCGACAAGGCTATTTTC
            10        20         30        40        50

            70        80        90       100       110       120
CCGAAATCCTTTTTCTATCGCCTGCGCAACCTCGCTGGCAGATAAACTTTCTTTATAAGA
   |||| || |
GACAAATACTATGATTTGATGAAAGCGATGGCGGGTTCCGTGGTGCATACCGGGGAAATC
60          70        80        90       100       110
```

```
SCORES
        97.8% identity in 46 bp overlap

                                        389       379       369
                                    GATACTTCTCACGGCGTCGGCGCACAACTT
                                    |||||||||||||||||||||||||||||
TGCATATTAAGGATCTGGCGAATCGCGCTGGATACTTCTCACGGCGTCGGCGCACAACTG
    340       350       360       370       380       390

  359       349       339       329       319       309
CCGCTCACAGCTGCGGTTATGGAGATGATGCAGGCACTGCGAACAGATGGTTTAGGAACG
|||||||||||||||
CCGCTCACAGCTGCGG
    400
```

Figure 14. FastA investigation of the sequences generated by the Universal M13 Forward (-20) and Universal M13 Reverse primers. Fasta investigation revealed identity in a 72bp overlap (62.5%) and a 46bp overlap (97.8%). The sequences generated by the Universal primers are homologous in a 15bp sequence identified as primer 1N (5'-GAACGCCAACGACGG).

```
Frame: (1)
 50.0% identity in 12 aa overlap

   110       120       130       140       150       160
SDSERTALNGEAKQLQKELDRISNTTTFGGRKLLDGSFGVASFQVGSAANEIISVGIDEM
                              |  ||:| ||::
AVPCWMPKRRWXWTATSSRASVLICILRIWRIALDTSHGVGAQLPLTAA
 90       100       110       120       130
```

```
Frame: (2)
 66.7% identity in 3 aa overlap

     150       160       170       180       190       200
GVASFQVGSAANEIISVGIDEMSAESLNGTYFKADGGGAVTAATASGTVDIAIGITGGSA
                              ||::
            AXTPTTVRCQXWWRRQGYFRQILXFDESDGGFRGAYRGNRCRXRHQT
                     10        20        30        40
```

```
Frame: (3)
 19.6% identity in 56 aa overlap

     190       200       210       220       230       240
TAATASGTVDIAIGITGGSAVNVKVDMKGNETAEQAAAKIAAAVNDANVGIGAFSDGDTI
                              :|:| :|::::  :|:: |:::  : ::::
RRYAVSDGGGDKAIFDKYYDLMKAMAGSVVHTGEIGAGNVTKLANQVIVALNIAAMSEAL
   10        20        30        40        50        60

     250       260       270       280       290       300
SYVSKAGKDGSGAITSAVSGVVIADTGSTGVGTAAGVAPSATAFAKTNDTVAKIDISTAK
: ::||| : :: :  |::| : ::|
TLATKAGVN-PDLVYQAIRGGLAGSTVLDAKAPMVMDRNFKPGFRIDLHIKDLANRAGYF
   70        80        90       100       110       120
```

Figure 15. TfastA investigation of nucleotide sequences (generated by the Universal M13 Forward -20 primer) of the insert of pKWII and the amino acid sequences of the PAK flagellin a-type gene. Tfasta investigation revealed identity in a 12aa overlap (Frame 1), 3aa overlap (Frame 2), 56aa overlap (Frame 3), 18aa overlap (Frame 4), 14aa overlap (Frame 5), and 38aa overlap (Frame 6).

```
Frame: (4)
 33.3% identity in 18 aa overlap

340       350       360       370       380       390
NIGENVSAARGRIEDTDFAAETANLTKNQVLQQAGTAILAQANQLPQSVLSLLPV
                                |:  ::: || :: ||:|
AAVVRRRREKYPARFARSLICRSIRKPGLKLRSITIGALASSTVLPASPPRIAXXTRSGL
    10        20        30        40        50        60
```

```
Frame: (5)
 21.4% identity in 14 aa overlap

        290       300       310       320       330       340
SATAFAKTNDTVAKIDISTAKALSRRAGDRTTAIKQIDASVPTSVAVQNRFDNTINNLKN
                                ::|:: |: : |:::
                          RSCERQLCADAVRSIQRDSPDPXYADQYGSPAXSCGPS
                                  10        20        30
```

```
Frame: (6)
 13.2% identity in 38 aa overlap

 290       300       310       320       330       340
KTNDTVAKIDISTAKALSRRAGDRTTAIKQIDASVPTSVAVQNRFDNTINNLKNIGENVS
                                | : :   :::::|:: :::: :::| : :
             AAVSGSCAPTPXEVSSAIRQILNMQINTEARLEVAVHHHRRFGIQHG
                          10        20        30        40

 350       360       370       380       390
AARGRIEDTDFAAETANLTKNQVLQQAGTAILAQANQLPQSVLSLLPV
:|| ::::
TARQSTANCLINQVRVNARFSCQRXRFXHRGNIQRHNDLICQFGDVTCTDFPGMHHGTRH
 50        60        70        80        90       100
```

Figure 15 (continued).

```
Frame: (1)
 27.8% identity in 18 aa overlap

      170       180       190       200       210       220
EMSAESLNGTYFKADGGGAVTAATASGTVDIAIGITGGSAVNVKVDMKGNETAEQAAAKI
                                  |||  :|:::: : |::
VWGDYDFHTYACYHMTPEGVSLIRPGFQARIAIRHPGTTSLTSNFDFRQFFVVARQGAMI
        50        60        70        80        90       100
```

```
Frame: (2)
 26.3% identity in 38 aa overlap

200       210       220       230       240       250
GGSAVNVKVDMKGNETAEQAAAKIAAAVNDANVGIGAFSDGDTISYVSKAGKDGSGAITS
                                  :::: ::|| : ::| ::| : ::||||
                AXTPTTATGTETYCASGKISRNPFSIACATSLADKLSLXESGAITI
                         10        20        30        40

260       270       280       290       300       310
AVSGVVIADTGSTGVGTAAGVAPSATAFAKTNDTVAKIDISTAKALSRRAGDRTTAIKQI
 ::  |:::
FIPMPVTTXRRRAFLLFGLDSRPGLQYAIRARRHXRVTSTFASFSXXHARALXSAVPKPS
```

```
Frame: (3)
 62.5% identity in 8 aa overlap

                10        20        30        40        50
      ALTVNTNIASLNTQPNLNNSSASLNTSLQRLSTGSRINSAKDDAAGLQIANRL
                                  | :|| ||:
QPEQKRTVHQEKFPEILFLSPAQPRWQINFLYKSLGRLRFSYLCLLPHDAGGRFSYSAWI
  10        20        30        40        50        60
```

Figure 16. TfastA investigation of nucleotide sequences (generated by Universal M13 Reverse primer) of the insert of pKWII and the amino acid sequences of the PAK flagellin a-type gene. Tfasta investigation revealed identity in an 18aa overlap (Frame 1), 38aa overlap (Frame 2), 8aa overlap (Frame 3), 13aa overlap (Frame 4), 3aa overlap (Frame 5), and 11aa overlap (Frame 6).

```
Frame: (4)
 38.5% identity in 13 aa overlap

240       250       260       270       280       290      299
 TISYVSKAGKDGSGAITSAVSGVVIADTGSTGVGTAAGVAPSATAFAKTNDTVAKIDIST
                                ||:|    :||::
 RXXRRARMAYCNPGLESRPNKRNALRRHVVTGIGMKIVIAPDSYKESLSASEVAQAIEKG
50        60        70        80        90       100
```

```
Frame: (5)
100.0% identity in 3 aa overlap

   110       120       130       140       150       160
DSERTALNGEAKQLQKELDRISNTTTFGGRKLLDGSFGVASFQVGSAANEIISVGIDEMS
                                |||
WRXCRHCEQMVXERRIIAPWRATTKNWRKSKLLVNDVVPGWRIAIRAWNPGRIRETPSGV
  20        30        40        50        60        70
```

```
Frame: (6)
 36.4% identity in 11 aa overlap

230       240       250       260       270       280      289
 VGIGAFSDGDTISYVSKAGKDGSGAITSAVSGVVIADTGSTGVGTAAGVAPSATAFAKTN
                                :||:: :||::
 RRRTTSAHSCGYGDDAGTANRWFRNGGSXRPGVLLRKTGESRSYSLMTSCPDGVLQSGPG
     10        20        30        40        50        60
```

Figure 16 (continued).

```
72.5% identity in 40 bp overlap

        330       340       350       360       370      379
TAAGGATCTGGCGAATCGCGCTGGATACTTCTCACGGCGTCGGCGC-ACAACTGCCGCTC
                                || |||||| ||||||    ||||| ||
CAGAGTCGCTGAACGGCACCTACTTCAAGGCTGACGGCGGCGGCGCGGTCACTGCTGC-A
    910       920       930       940       950       960

380      389
ACAGCTGCGG
|| ||| |||
ACCGCTTCGGGCACCGTCGACATCGCGATCGGCATCACCGGCGGCAGCGCCGTGAACGTC
     970       980       990      1000      1010      1020
```

Figure 17. FastA investigation of the nucleotide sequences (generated by the Universal M13 Forward -20 primer) of the insert of pKWII and the PAK flagellin a-type gene. Fasta investigation revealed identity (72.5%) in bases 934-972.

```
     70.0% identity in 40 bp overlap

                          369       359        349       339
                        GATACTTCTCACGGCGTCGGCGC-ACAACTTCCGCTC
                                || |||||| ||||||    ||| | ||
CAGAGTCGCTGAACGGCACCTACTTCAAGGCTGACGGCGGCGGCGCGGTCACTGCTGC-A
    910       920       930       940       950       960

      329       319       309       299       289       279
ACAGCTGCGGTTATGGAGATGATGCAGGCACTGCGAACAGATGGTTTAGGAACGGCGGAT
|| ||| |||
ACCGCTTCGGGCACCGTCGACATCGCGATCGGCATCACCGGCGGCAGCGCCGTGAACGTC
     970       980       990      1000      1010      1020
```

Figure 18. FastA investigation of the nucleotide sequences (generated by the Universal M13 Reverse primer) of the insert of pKWII and the PAK flagellin a-type gene. Fasta investigation revealed identity (70.0%) in bases 934-972.

```
>Universal M13 Forward (-20) generated sequences minus 1N
1TACGCTGTCAGTGATGGTGGCGGCGACAAGGCTATTTTCGACAAATACTA

51TGATTTGATGAAAGCGATGGCGGGTTCCGTGGTGCATACCGGGGAAATCG

101GTGCAGGTAACGTCACCAAACTGGCAAATCAGGTCATTGTGGCGCTGAAT

151ATTGCCGCGATGTCAGAAGCGTTAACGCTGGCAACTAAAGCGGGCGTTAA

201CCCGGACCTGGTTTATCAGGCAATTCGCGGTGGACTGGCGGGCAGTACCG

251TGCTGGATGCCAAAGCGCCGATGGTGATGGACCGCAACTTCAAGCCGGGC

301TTCCGTATTGATCTGCATATTAAGGATCTGGCGAATCGCGCTGGATACTT

351CTCACGGCGTCGGCGCACAACTGCCGCTCACAGCTGCGG
```

```
Database:                    PDB+GBupdate+GenBank+EMBLupdate+EMBL
           520,383 sequences; 367,017,413 total letters.
Searching..................................................done


Sequences producing High-scoring Segment Pairs:

dbj|D90212|ECORNPBW E.coli rnpB gene and ORFs. >emb|D9021...

gb|U18997|ECOUW67   Escherichia coli K-12 chromosomal reg...
dbj|D21153|ECOPYU46 E.coli gene for unknown product, part...
```

Figure 19. BLAST (Netscape nucleotide database program) sequence matches with the sequences (generated by the Universal M13 Forward -20 primer) of the insert of pKWII. BLAST database searches matched the sequences of the insert of pKWII with sequences of the bacterium *Escherichia coli.*

```
>dbj|D90212|ECORNPBW E.coli rnpB gene and ORFs. >emb|D90212|ECRNPBW E.coli rnpB
            gene and ORFs.
            Length = 4434

  Plus Strand HSPs:

 Score = 1590 (439.3 bits), Expect = 5.8e-146, Sum P(3) = 5.8e-146
 Identities = 318/318 (100%), Positives = 318/318 (100%), Strand = Plus / Plus

Query:    19 GGCGGCGACAAGGCTATTTTCGACAAATACTATGATTTGATGAAAGCGATGGCGGGTTCC 78
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   951 GGCGGCGACAAGGCTATTTTCGACAAATACTATGATTTGATGAAAGCGATGGCGGGTTCC 1010

Query:    79 GTGGTGCATACCGGGGAAATCGGTGCAGGTAACGTCACCAAACTGGCAAATCAGGTCATT 138
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1011 GTGGTGCATACCGGGGAAATCGGTGCAGGTAACGTCACCAAACTGGCAAATCAGGTCATT 1070

Query:   139 GTGGCGCTGAATATTGCCGCGATGTCAGAAGCGTTAACGCTGGCAACTAAAGCGGGCGTT 198
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1071 GTGGCGCTGAATATTGCCGCGATGTCAGAAGCGTTAACGCTGGCAACTAAAGCGGGCGTT 1130

Query:   199 AACCCGGACCTGGTTTATCAGGCAATTCGCGGTGGACTGGCGGGCAGTACCGTGCTGGAT 258
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1131 AACCCGGACCTGGTTTATCAGGCAATTCGCGGTGGACTGGCGGGCAGTACCGTGCTGGAT 1190

Query:   259 GCCAAAGCGCCGATGGTGATGGACCGCAACTTCAAGCCGGGCTTCCGTATTGATCTGCAT 318
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1191 GCCAAAGCGCCGATGGTGATGGACCGCAACTTCAAGCCGGGCTTCCGTATTGATCTGCAT 1250

Query:   319 ATTAAGGATCTGGCGAAT 336
             ||||||||||||||||||
Sbjct:  1251 ATTAAGGATCTGGCGAAT 1268

 Score = 260 (71.8 bits), Expect = 5.8e-146, Sum P(3) = 5.8e-146
 Identities = 52/52 (100%), Positives = 52/52 (100%), Strand = Plus / Plus

Query:   338 GCGCTGGATACTTCTCACGGCGTCGGCGCACAACTGCCGCTCACAGCTGCGG 389
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1269 GCGCTGGATACTTCTCACGGCGTCGGCGCACAACTGCCGCTCACAGCTGCGG 1320

 Score = 100 (27.6 bits), Expect = 5.8e-146, Sum P(3) = 5.8e-146
 Identities = 20/20 (100%), Positives = 20/20 (100%), Strand = Plus / Plus

Query:     1 TACGCTGTCAGTGATGGTGG 20
             ||||||||||||||||||||
Sbjct:   932 TACGCTGTCAGTGATGGTGG 951


>gb|U18997|ECOUW67 Escherichia coli K-12 chromosomal region from 67.4 to 76.0
            minutes.
            Length = 372,438

  Minus Strand HSPs:
```

Figure 19 (continued).

```
 Score = 1590 (439.3 bits), Expect = 7.3e-142, Sum P(3) = 7.3e-142
 Identities = 318/318 (100%), Positives = 318/318 (100%), Strand = Minus / Plus

Query:    336 ATTCGCCAGATCCTTAATATGCAGATCAATACGGAAGCCCGGCTTGAAGTTGCGGTCCAT 277
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  52779 ATTCGCCAGATCCTTAATATGCAGATCAATACGGAAGCCCGGCTTGAAGTTGCGGTCCAT 52838

Query:    276 CACCATCGGCGCTTTGGCATCCAGCACGGTACTGCCCGCCAGTCCACCGCGAATTGCCTG 217
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  52839 CACCATCGGCGCTTTGGCATCCAGCACGGTACTGCCCGCCAGTCCACCGCGAATTGCCTG 52898

Query:    216 ATAAACCAGGTCCGGGTTAACGCCCGCTTTAGTTGCCAGCGTTAACGCTTCTGACATCGC 157
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  52899 ATAAACCAGGTCCGGGTTAACGCCCGCTTTAGTTGCCAGCGTTAACGCTTCTGACATCGC 52958

Query:    156 GGCAATATTCAGCGCCACAATGACCTGATTTGCCAGTTTGGTGACGTTACCTGCACCGAT 97
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  52959 GGCAATATTCAGCGCCACAATGACCTGATTTGCCAGTTTGGTGACGTTACCTGCACCGAT 53018

Query:     96 TTCCCCGGTATGCACCACGGAACCCGCCATCGCTTTCATCAAATCATAGTATTTGTCGAA 37
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  53019 TTCCCCGGTATGCACCACGGAACCCGCCATCGCTTTCATCAAATCATAGTATTTGTCGAA 53078

Query:     36 AATAGCCTTGTCGCCGCC 19
              ||||||||||||||||||
Sbjct:  53079 AATAGCCTTGTCGCCGCC 53096

 Score = 260 (71.8 bits), Expect = 7.3e-142, Sum P(3) = 7.3e-142
 Identities = 52/52 (100%), Positives = 52/52 (100%), Strand = Minus / Plus

Query:    389 CCGCAGCTGTGAGCGGCAGTTGTGCGCCGACGCCGTGAGAAGTATCCAGCGC 338
              ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  52727 CCGCAGCTGTGAGCGGCAGTTGTGCGCCGACGCCGTGAGAAGTATCCAGCGC 52778

 Score = 100 (27.6 bits), Expect = 7.3e-142, Sum P(3) = 7.3e-142
 Identities = 20/20 (100%), Positives = 20/20 (100%), Strand = Minus / Plus

Query:     20 CCACCATCACTGACAGCGTA 1
              ||||||||||||||||||||
Sbjct:  53096 CCACCATCACTGACAGCGTA 53115

 Score = 83 (22.9 bits), Expect = 2.1e-10, Sum P(2) = 2.1e-10
 Identities = 23/31 (74%), Positives = 23/31 (74%), Strand = Minus / Plus

Query:     183 TGCCAGCGTTAACGCTTCTGACATCGCGGCA 153
               ||||||||||||||| ||    | ||| | |
Sbjct:  150149 TGCCAGCGTTAACGCGTCCTGTAACGCAGGA 150179

 Score = 79 (21.8 bits), Expect = 7.5e-10, Sum P(3) = 7.5e-10
 Identities = 19/23 (82%), Positives = 19/23 (82%), Strand = Minus / Plus

Query:     335 TTCGCCAGATCCTTAATATGCAG 313
               |||||||||||  ||| ||| ||
Sbjct:  117812 TTCGCCAGATCGATAAGATGGAG 117834


>dbj|D21153|ECOPYU46 E.coli gene for unknown product, partial cds.
           >emb|D21153|ECPYU15 E.coli gene for unknown product, partial cds.
           Length = 300
```


Figure 19 (continued).

```
 Plus Strand HSPs:

 Score = 317 (87.6 bits), Expect = 1.0e-16, P = 1.0e-16
 Identities = 141/238 (59%), Positives = 141/238 (59%), Strand = Plus / Plus

Query:    91 GGGGAAATCGGTGCAGGTAACGTCACCAAACTGGCAAATCAGGTCATTGTGGCGCTGAAT 150
             || |  | ||| |  ||| |   |  |||| |||||||||| | || |||||||| |||
Sbjct:    63 GGCGGTAACGGCGATGGTCAAACCTGCAAAGTGGCAAATCAGATTATCGTGGCGCTCAAT 122

Query:   151 ATTGCCGCGATGTCAGAAGCGTTAACGCTGGCAACTAAAGCGGGCGTTAACCCGGACCTG 210
             ||||  ||| | || |||||  |     | ||  | ||||| || |   ||||||  |
Sbjct:   123 ATTGAAGCGGTTTCTGAAGCCCTGCTATTTGCTTCAAAAGCCGGTGCGGACCCGGTACGT 182

Query:   211 GTTTATCAGGCAATTCGCGGTGGACTGGCGGGCAGTACCGTGCTGGATGCCAAAGCGCCG 270
             ||    |||||  |    || ||  | ||   |       | ||||| |   | |    |
Sbjct:   183 GTGCGCCAGGCGCTGATGGGCGGCTTTGCTTCCTCACGTATTCTGGAAGTTCATGGCGAG 242

Query:   271 ATGGTGATGGACCGCAACTTCAAGCCGGGCTTCCGTATTGATCTGCATATTAAGGATC 328
                 ||||  | |||| ||| || |||||||||   || | ||||||    || ||||
Sbjct:   243 CGTATGATTAAACGCACCTTTAATCCGGGCTTCAAAATCGCTCTGCACCAGAAAGATC 300
```


Figure 19 (continued).

```
>Universal M13 Reverse generated sequences minus 1N
1CAACCGGAACAGAAACGTACTGTGCATCAGGAAAAATTTCCCGAAATCCT

51TTTTCTATCGCCTGCGCAACCTCGCTGGCAGATAAACTTTCTTTATAAGA

101GTCTGGGGCGATTACGATTTTCATACCTATGCCTGTTACCACATGACGCC

151GGAGGGCGTTTCTCTTATTCGGCCTGGATTCCAGGCCCGGATTGCAATAC

201GCCATCCGGGCACGACGTCATTAACGAGTAACTTCGACTTTCGCCAGTTT

251TTCGTAGTAGCACGCCAGGGCGCTATGATCCGCCGTTCCTAAACCATCTG

301TTCGCAGTGCCTGCATCATCTCCATAACCGCAGCTGTGAGCGGAAGTTGT
```

```
Database:                    PDB+GBupdate+GenBank+EMBLupdate+EMBL
           520,383 sequences; 367,017,413 total letters.
Searching..................................................done


Sequences producing High-scoring Segment Pairs:

dbj|D90212|ECORNPBW E.coli rnpB gene and ORFs. >emb|D9021...
gb|U18997|ECOUW67   Escherichia coli K-12 chromosomal reg...
gb|U32694|HIU32694  Haemophilus influenzae tRNA-Gly-A, tR...
```

Figure 20. BLAST (Netscape nucleotide database program) sequence match with the sequences (generated by the Universal M13 Reverse primer) of the insert of pKWII. BLAST database searches matched the sequences of the insert of pKWII with sequences of the bacteria *Escherichia coli* and *Haemophilus influenzae*.

```
>dbj|D90212|ECORNPBW E.coli rnpB gene and ORFs. >emb|D90212|ECRNPBW E.coli rnpB
            gene and ORFs.
            Length = 4434

  Minus Strand HSPs:

 Score = 1732 (478.6 bits), Expect = 1.7e-136, P = 1.7e-136
 Identities = 348/350 (99%), Positives = 348/350 (99%), Strand = Minus / Plus

Query:   350 ACAACTTCCGCTCACAGCTGCGGTTATGGAGATGATGCAGGCACTGCGAACAGATGGTTT 291
             |||||| |||||||||||||||||||||||||||||||||||||||||||| |||||||||||
Sbjct:  1298 ACAACTGCCGCTCACAGCTGCGGTTATGGAGATGATGCAGGCACTGCGAGCAGATGGTTT 1357

Query:   290 AGGAACGGCGGATCATAGCGCCCTGGCGTGCTACTACGAAAAACTGGCGAAAGTCGAAGT 231
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1358 AGGAACGGCGGATCATAGCGCCCTGGCGTGCTACTACGAAAAACTGGCGAAAGTCGAAGT 1417

Query:   230 TACTCGTTAATGACGTCGTGCCCGGATGGCGTATTGCAATCCGGGCCTGGAATCCAGGCC 171
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1418 TACTCGTTAATGACGTCGTGCCCGGATGGCGTATTGCAATCCGGGCCTGGAATCCAGGCC 1477

Query:   170 GAATAAGAGAAACGCCCTCCGGCGTCATGTGGTAACAGGCATAGGTATGAAAATCGTAAT 111
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1478 GAATAAGAGAAACGCCCTCCGGCGTCATGTGGTAACAGGCATAGGTATGAAAATCGTAAT 1537

Query:   110 CGCCCCAGACTCTTATAAAGAAAGTTTATCTGCCAGCGAGGTTGCGCAGGCGATAGAAAA 51
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1538 CGCCCCAGACTCTTATAAAGAAAGTTTATCTGCCAGCGAGGTTGCGCAGGCGATAGAAAA 1597

Query:    50 AGGATTTCGGGAAATTTTTCCTGATGCACAGTACGTTTCTGTTCCGGTTG 1
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1598 AGGATTTCGGGAAATTTTTCCTGATGCACAGTACGTTTCTGTTCCGGTTG 1647


>gb|U18997|ECOUW67 Escherichia coli K-12 chromosomal region from 67.4 to 76.0
            minutes.
            Length = 372,438

  Plus Strand HSPs:

 Score = 1732 (478.6 bits), Expect = 2.2e-136, P = 2.2e-136
 Identities = 348/350 (99%), Positives = 348/350 (99%), Strand = Plus / Plus

Query:     1 CAACCGGAACAGAAACGTACTGTGCATCAGGAAAAATTTCCCGAAATCCTTTTTCTATCG 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 52400 CAACCGGAACAGAAACGTACTGTGCATCAGGAAAAATTTCCCGAAATCCTTTTTCTATCG 52459

Query:    61 CCTGCGCAACCTCGCTGGCAGATAAACTTTCTTTATAAGAGTCTGGGGCGATTACGATTT 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 52460 CCTGCGCAACCTCGCTGGCAGATAAACTTTCTTTATAAGAGTCTGGGGCGATTACGATTT 52519

Query:   121 TCATACCTATGCCTGTTACCACATGACGCCGGAGGGCGTTTCTCTTATTCGGCCTGGATT 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 52520 TCATACCTATGCCTGTTACCACATGACGCCGGAGGGCGTTTCTCTTATTCGGCCTGGATT 52579

Query:   181 CCAGGCCCGGATTGCAATACGCCATCCGGGCACGACGTCATTAACGAGTAACTTCGACTT 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

Figure 20 (continued).

```
Sbjct:  52580 CCAGGCCCGGATTGCAATACGCCATCCGGGCACGACGTCATTAACGAGTAACTTCGACTT 52639

Query:    241 TCGCCAGTTTTTCGTAGTAGCACGCCAGGGCGCTATGATCCGCCGTTCCTAAACCATCTG 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  52640 TCGCCAGTTTTTCGTAGTAGCACGCCAGGGCGCTATGATCCGCCGTTCCTAAACCATCTG 52699

Query:    301 TTCGCAGTGCCTGCATCATCTCCATAACCGCAGCTGTGAGCGGAAGTTGT 350
               |||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct:  52700 CTCGCAGTGCCTGCATCATCTCCATAACCGCAGCTGTGAGCGGCAGTTGT 52749


>gb|U32694|HIU32694 Haemophilus influenzae tRNA-Gly-A, tRNA-Cys, tRNA-Leu-A,
            tRNA-Lys-A, thrC, thrB, thrA genes from bases 90826 to 101117 of
            the complete genome.
            Length = 10,292

  Plus Strand HSPs:

 Score = 204 (56.4 bits), Expect = 6.5e-07, P = 6.5e-07
 Identities = 76/120 (63%), Positives = 76/120 (63%), Strand = Plus / Plus

Query:     15 ACGTACTGTGCATCAGGAAAAATTTCCCGAAATCCTTTTTCTATCGCCTGCGCAACCTCG 74
              || || |  ||||| || || |         || ||| |||| || ||    || || ||
Sbjct:   7562 ACATAGTCCGCATCGGGGAATACACGTTTGAAGCCTGTTTCAATGGCGGTAGCGACTTCA 7621

Query:     75 CTGGCAGATAAACTTTCTTTATAAGAGTCTGGGGCGATTACGATTTTCATACCTATGCCT 134
                || |  |||||||||||| |||| || || || || || | ||||||| | |  |||
Sbjct:   7622 AGTGCGGTCAAACTTTCTTTAAAAGAATCAGGTGCAATCACAAATTTCATAACAACTCCT 7681
```

Figure 20 (continued).

Attempts to identify flagellin DNA in clone pKW52 by Southern hybridization analysis.

The Southern hybridization method was used in addition to PCR to locate the flagellin b-type gene, because DNA and oligonucleotide probes have been used successfully to locate and identify genes in *Pseudomonas aeruginosa* **(refer to Chapter 4)** strains PAK and PAO1 (Farinha *et al.*, 1993; Totten *et al.*, 1990; Strom *et al.*, 1986). Totten *et al.*, 1990 identified clones containing the flagellin a-type gene using the 1.7kb *EcoRI-HindIII* fragment (PAK flagellin a-type gene) of clone pPT218. A 15-mer oligonucleotide derived from the N-terminal sequence of the PAK flagellin a-type gene and the entire PAK flagellin a-type gene were radiolabeled, and used as probes to locate and identify the flagellin b-type gene in pKW52 and pKWII, respectively. The oligonucleotide and DNA probes were derived from the PAK flagellin a-type gene, because it was hypothesized that the sequence similiarity between the PAK flagellin a-type gene and the PAO1 flagellin b-type gene. Therefore, the PAK flagellin a-type gene would be an ideal probe to locate the flagellin b-type gene.

Clone pKW52 was digested with enzymes *EcoRI*, *KpnI*, *XhoI*, or *PstI*. Previous studies (Wintenberg *et al.*, 1989) locating the flagellin b-type gene on a restriction map of clone pKW52 used the restriction endonucleases indicated above. The restriction fragments and undigested DNA were separated electrophoretically on an agarose gel, denatured, and transferred onto nylon membrane. After prehybridization to prevent nonspecific binding of the probe, the membrane was hybridized either to the 1.7kb flagellin (a-type) gene or to the 1N probe. The PAK flagellin a-type gene was designated 1.7kb flagellin (a-type) gene probe and the 15-mer oligonucleotide was designated probe 1N. The membrane was washed several times and exposed to Kodak XRP-1 X-Ray film. To test the validity of the Southern hybridization experiments clone pPT218 (flagellin a-type) was used as a positive control. Self-recognition of the flagellin a-type gene fragment by hybridization with the 1.7kb flagellin (a-type) gene probe under high stringency conditions is shown in **Figures 21 (lanes 11, 12, 13), 22 (lanes 3, 6), 23 (lane 4), 7 (lane 2)**. Based on consistent self-recognition of the 1.7kb flagellin (a-type) gene probe to the flagellin a-type gene under high and

Figure 21A. Agarose gel electrophoresis of restriction digests of clone pKW52. **Lane (1)** 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(2)** No sample loaded in the well, **(3)** Clone pKW52 cosmid DNA uncut, **(4)** Clone pKW52 cosmid DNA cut with enzyme *EcoRI*, **(5)** Clone pKW52 cosmid DNA cut with enzyme *XhoI*, **(6)** Clone pKW52 cosmid DNA cut with enzyme *PstI*, **(7)** Clone pKW52 cosmid DNA cut with enzyme *KpnI*, **(8)** No sample loaded in the well, **(9)** 1 µl 1kb Molecular Weight Marker (1:20 dilution of stock [250µg/242.7µl]), **(10)** No sample loaded in the well, **(11)** Clone pPT218 plasmid DNA cut with enzymes *EcoRI* and *HindIII*, **(12)** Clone pPT218 plasmid DNA cut with enzymes *EcoRI* and *XbaI*, **(13)** Clone pPT218 plasmid DNA cut with enzymes *StyI* and *HindIII*, **(14)** No sample loaded in the well, **(15)** No sample loaded in the well.

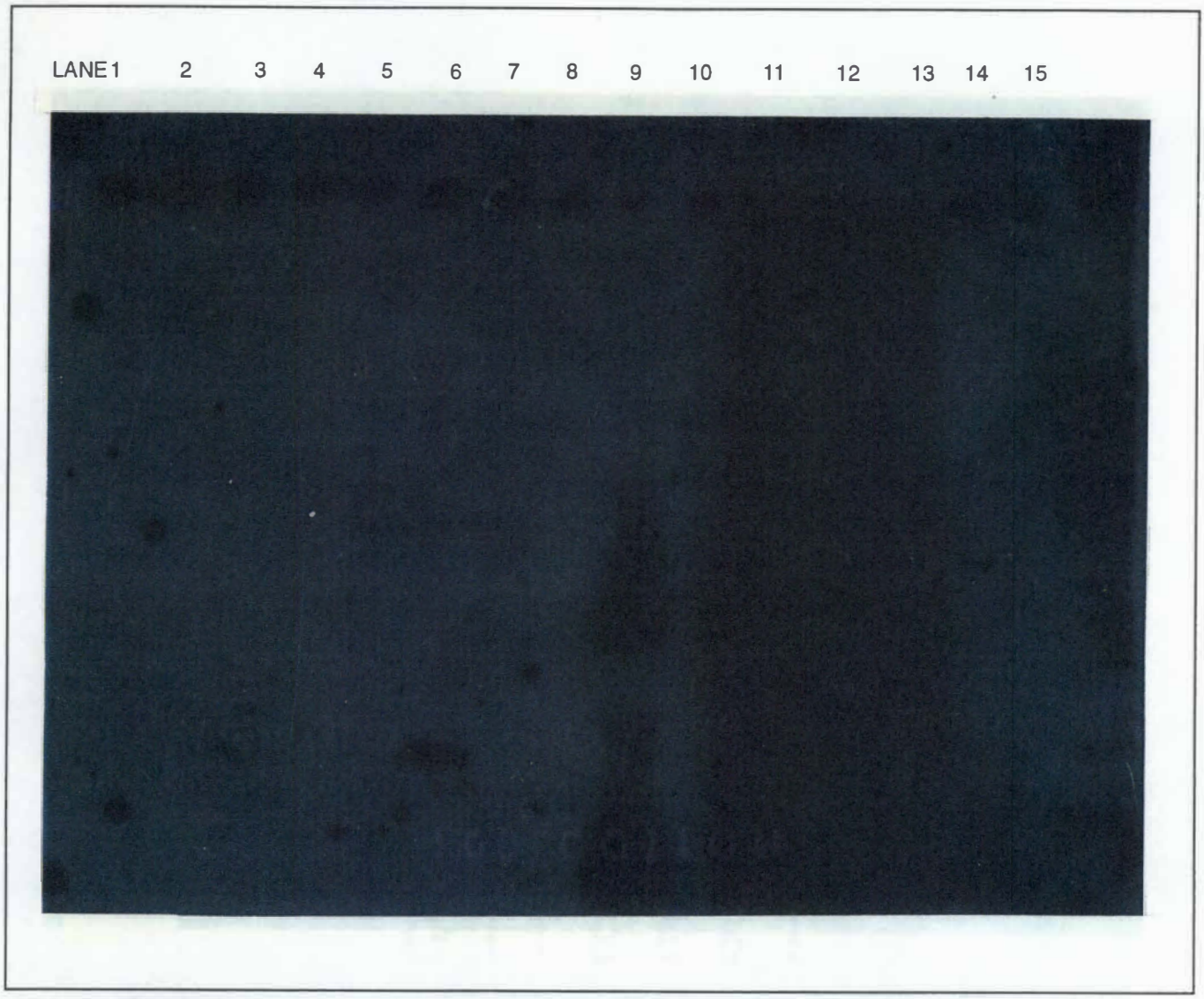

Figure 21B. Autoradiogram of Figure 21A. Southern hybridization with the flagellin (a-type gene) probe under low stringency hybridization conditions. **Lane (1)** 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(2)** No sample loaded in the well, **(3)** Clone pKW52 cosmid DNA uncut, **(4)** Clone pKW52 cosmid DNA cut with enzyme *EcoRI*, **(5)** Clone pKW52 cosmid DNA cut with enzyme *XhoI*, **(6)** Clone pKW52 cosmid DNA cut with enzyme *PstI*, **(7)** Clone pKW52 cosmid DNA cut with enzyme *KpnI*, **(8)** No sample loaded in the well, **(9)** 1 µl 1kb Molecular Weight Marker (1:20 dilution of stock [250µg/242.7µl]), **(10)** No sample loaded in the well, **(11)** Clone pPT218 plasmid DNA cut with enzymes *EcoRI* and *HindIII*, **(12)** Clone pPT218 plasmid DNA cut with enzymes *EcoRI* and *XbaI*, **(13)** Clone pPT218 plasmid DNA cut with enzymes *StyI* and *HindIII*, **(14)** No sample loaded in the well, **(15)** No sample loaded in the well.

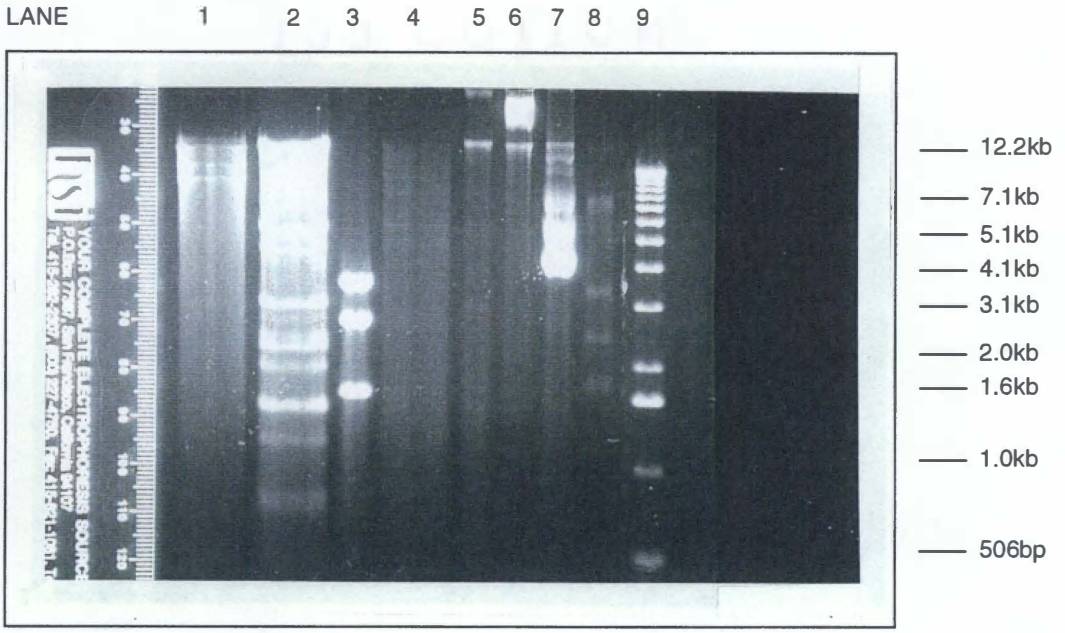

Figure 22A. Agarose gel electrophoresis of clones pKW52 and pPT218. Lane (1) Clone pKW52 cosmid DNA cut with *EcoRI*, **(2)** Clone pKW52 cosmid DNA cut with *PstI*, **(3)** Clone pPT218 plasmid DNA cut with *EcoRI-HindIII*, **(4)** Clone pKWII plasmid DNA cut with *EcoRI*, **(5)** Clone pKW52 cosmid DNA uncut, **(6)** Clone pPT218 plasmid DNA uncut, **(7)** Clone pKWII plasmid DNA uncut, **(8)** 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(9)** 1 µl 1kb Molecular Weight Marker (1:20 dilution of stock [250µg/242.7µl]).

Figure 22B. Autoradiogram of Figure 22A. Southern hybridization with the flagellin (a-type gene) probe under high stringency hybridization conditions. Lane (1) Clone pKW52 cosmid DNA cut with *EcoRI*, **(2)** Clone pKW52 cosmid DNA cut with *PstI*, **(3)** Clone pPT218 plasmid DNA cut with *EcoRI* and *HindIII*, **(4)** Clone pKWII plasmid DNA cut with *EcoRI*, **(5)** Clone pKW52 cosmid DNA uncut, **(6)** Clone pPT218 plasmid DNA uncut, **(7)** Clone pKWII plasmid DNA uncut, **(8)** 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(9)** 1 µl 1kb Molecular Weight Marker (1:20 dilution of stock [250µg/242.7µl]).

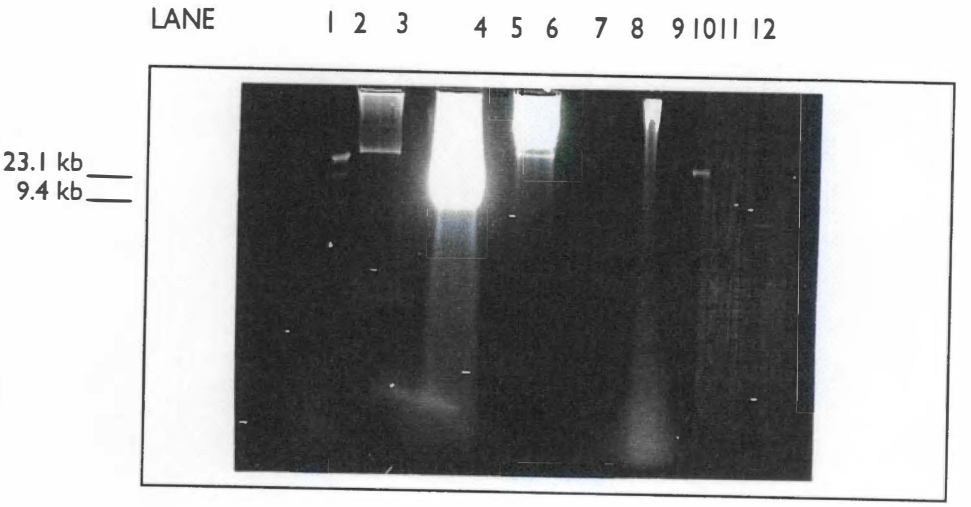

Figure 23A. Agarose gel electrophoresis of undigested pKW52 cosmid DNA. Lane (1) 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(2)** Alkaline lysed PA103 cells, **(3)** No sample loaded in the well, **(4)** Clone pPT218 plasmid DNA uncut, **(5)** No sample loaded in the well, **(6)** Clone pKW52 cosmid DNA uncut, **(7)** No sample loaded in the well, **(8)** No sample loaded in the well, **(9)** Alkaline lysed PAO1 cells, **(10)** No sample loaded in the well, **(11)** 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(12)** No sample loaded in the well.

Figure 23B. Autoradiogram of Figure 23A. Southern hybridization with the flagellin (a-type gene) probe under high stringency hybridization conditions. **Lane (1)** 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(2)** Alkaline lysed PA103 cells, **(3)** No sample loaded in the well, **(4)** Clone pPT218 plasmid DNA uncut, **(5)** No sample loaded in the well, **(6)** Clone pKW52 cosmid DNA uncut, **(7)** No sample loaded in the well, **(8)** No sample loaded in the well, **(9)** Alkaline lysed PAO1 cells, **(10)** No sample loaded in the well, **(11)** 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(12)** No sample loaded in the well.

low stringency hybridization conditions, it was concluded that clone pPT218 was an appropriate probe to validate the methodology used.

No DNA fragments of pKW52 digested with the restriction endonucleases indicated above were recognized by the 1.7kb flagellin (a-type) gene probe under low stringency hybridization conditions **(Figure 21A agarose gel, lanes 4, 5, 6, 7 ; Figure 21B autoradiogram, lanes 4, 5, 6, 7)**. Nonspecific binding of the 1.7kb flagellin (a-type) gene probe to various restriction fragments of pKW52 was observed. It was hypothesized that the hybridization of the 1.7kb flagellin (a-type) gene probe was nonspecific, because hybridization of the probe to the 1kb molecular weight marker was observed. In support of the hypothesis that the hybridization of the 1.7kb flagellin (a-type) gene probe to the restriction fragments of pKW52 was nonspecific, DNA of the restriction endonuclease pattern of pKW52 were not recognized by hybridization with the 1.7kb flagellin (a-type) gene probe under high stringency conditions as shown in **Figure 22A agarose gel, lanes 1, 2; Figure 22B autoradiogram, lanes 1, 2**. DNA fragments of pKW52 digested with the restriction endonuclease *KpnI* were not recognized by probe 1N under high stringency hybridization conditions **(Figure 7 agarose gel, lane 4; Figure 24 autoradiogram, lane 4)**.

DNA sequences of undigested pKW52 were not recognized by the 1.7kb flagellin (a-type) gene probe under high stringency hybridization conditions **(Figure 23A agarose gel, lane 6; Figure 23B autoradiogram, lane 6)**. Nonspecific binding of the 1.7kb flagellin (a-type) gene probe to undigested pKW52 was observed. The hybridization of the 1.7kb flagellin (a-type) gene probe to undigested pKW52 was concluded as nonspecific, because hybridization of the probe was observed in lanes **(Figure 23B autoradiogram, lanes 3, 5, 10, 12)** with no sample loaded in them. In addition, hybridization of the 1.7kb flagellin (a-type) gene probe was observed in **lanes 2** and **9** of **Figure 23B (autoradiogram)**. An alkaline lysis prep used to obtain chromosomal DNA was mistakenly done on PA103 and PA01 cells. Therefore, binding of the flagellin gene probe to samples loaded in **lanes 2** and **9** was unclear. Also, nonspecific binding of the flagellin probe to undigested pKW52 was observed in other Southern hybridization experiments as seen in **Figures 22 (22B autoradiogram, lane 5)**. In **Figure 22B (lane 5)**, the flagellin (a-type) gene probe appeared to hybridize to pKW52 DNA loaded in **lane 5**. It is hypothesized that the intense signal

Figure 24. Autoradiogram of Figure 7. Southern hybridization with probe 1N under high stringency hybridization conditions. Lane (1) 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(2)** Clone pPT218 plasmid DNA cut with *EcoRI* and *HindIII*, **(3)** PCR amplification products of clone pPT218, **(4)** Clone pKW52 cosmid DNA uncut, **(5)** Clone pKW52 cosmid DNA cut with *KpnI*, **(6)** PCR amplification products of clone pKW52, **(7)** Clone pKWII plasmid DNA cut with *EcoRI*, **(8)** 1 µl 1kb Molecular Weight Marker (1:20 dilution of stock [250µg/242.7µl]).

produced by the control (pPT218) was in close proximity to the lane loaded with pKW52; therefore, it appeared (experimental artifact) the flagellin (a-type) gene probe binded to pKW52. Furthermore, the pPT218 sample may have spilled (while loading the agarose gel) into an adjacent well. It had been reported that clone pKW52 contained the PAO1 flagellin b-type gene (Kelly-Wintenberg *et al.*, 1989). Yet, Southern hybridization experiments with the PAK flagellin (a-type) gene probe and probe 1N did not locate complementary sequences in pKW52.

Southern hybridization of pKWII.

Clone pKWII obtained by cloning the 600bp PCR amplified fragment of clone pKW52 was digested with enzyme *EcoRI*. Self-recognition of the insert of pKWII by hybridization with the 600bp gene probe under high stringency conditions is shown in **Figure 7 agarose gel, lane 6; Figure 25 autoradiogram, lane 6**. To test the validity of the Southern hybridization experiments the 600bp PCR amplified fragment of pKW52 was used as a positive control. DNA of the 600bp fragment was recognized by hybridization with the 600bp gene probe under high stringency conditions **(Figure 7 agarose gel, lane 5; Figure 25 autoradiogram, lane 5)**. Based on consistent self-recognition of the 600bp probe to the 600bp PCR amplified fragment and the 1.3kb insert of pKWII, it was concluded that the 600bp fragment was an appropriate probe to validate the methodology used.

Even though it was hypothesized based on size that the 600bp fragment was the flagellin b-type gene, the Southern hybridization results did not support that hypothesis. DNA of the 600bp fragment was not recognized by hybridization with the 1.7kb flagellin (a-type) gene probe under high stringency conditions **(Figure 7 agarose gel, lane 5; Figure 26 autoradiogram, lane 5)**. DNA of the 1.3kb insert of clone pKWII was not recognized by hybridization with the 1.7kb flagellin (a-type) gene probe under high stringency conditions **(Figure 7 agarose gel, lane 6; Figure 26 autoradiogram, lane 6)**. DNA of the 600bp fragment and the 1.3kb insert of pKWII was recognized by hybridization with probe 1N under high stringency conditions **(Figure 7 agarose gel electrophoresis, lane 5, 6; Figure 24 autoradiogram, lane 5, 6)**. However, the

Figure 25. Autoradiogram of Figure 7. Southern hybridization with the 600bp PCR amplified fragment of pKW52 under high stringency hybridization conditions. Lane (1) 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(2)** Clone pPT218 plasmid DNA cut with *EcoRI* and *HindIII*, **(3)** PCR amplification products of clone pPT218, **(4)** Clone pKW52 cosmid DNA uncut, **(5)** Clone pKW52 cosmid DNA cut with *KpnI*, **(6)** PCR amplification products of clone pKW52, **(7)** Clone pKWII plasmid DNA cut with *EcoRI*, **(8)** 1 µl 1kb Molecular Weight Marker (1:20 dilution of stock [250µg/242.7µl]).

Figure 26. Autoradiogram of Figure 7. Southern hybridization with the flagellin (a-type gene) probe under high stringency hybridization conditions. **Lane (1)** 1 µl Lambda *HindIII* Molecular Weight Marker (1:20 dilution of stock [200µg/500µl]), **(2)** Clone pPT218 plasmid DNA cut with *EcoRI* and *HindIII*, **(3)** PCR amplification products of clone pPT218, **(4)** Clone pKW52 cosmid DNAuncut, **(5)** Clone pKW52 cosmid DNA cut with *KpnI*, **(6)** PCR amplification products of clone pKW52, **(7)** Clone pKWII plasmid DNA cut with *EcoRI*, **(8)** 1 µl 1kb Molecular Weight Marker (1:20 dilution of stock [250µg/242.7µl]).

PAO1 flagellin b-type gene have an identical 12 amino acid sequence (Anderson *et al.*, unpublished report). In addition, studies have shown that the (N- and) C- terminal amino acid sequences of bacteria flagellin are highly conserved (Totten *et al.*, 1989). However, the Southern hybridization results **(Figures 7A, 21-24)** presence did not show a nucleotide sequence similarity between the hypothetical PAO1 flagellin b-type gene and the PAK flagellin a-type gene. The Southern hybridization results of this thesis indicated that the sequences of the PAK flagellin a-type gene and the sequences of clone pKW52 are not similar. Wintenberg *et al* (1989) reported that clone pKW52 contained the PAO1 flagellin b-type gene.

In spite of considerable care in designing and executing the Southern hybridization experiments, the flagellin (a-type) gene probe and probe 1N did not appear to be ideal probes to locate the flagellin b-type gene in clone pKW52. Additional southern hybridization analysis of pKW52 with other probes may locate the flagellin b-type gene. An analogous approach reported by Farinha *et al.*, 1993 could be employed in future approaches including PCR. They used oligonucleotides and double-stranded DNA probes in locating and identifying virulence-associated genes (*pilA*, *pilR*, *fliA*, and *fliC*) on large DNA fragments of *Pseudomonas aeruginosa* PAO1 (flagellin b-type). The following probes were used in the Farinha *et al* 1993 studies: an oligonucleotide derived from the *rpoN* gene sequence of *P. putida*, the flagellin structural gene *fliC* (PAK flagellin a-type), and the minor sigma factor gene *fliA*. The *fliC* gene encodes the flagellin subunits of *P. aeruginosa*. The *fliA* is a member of the σ^{28} class of sigma factors necessary for the expression of motility and chemotaxis genes in bacteria. Provided that these genes are located in the region of the structural gene (*fliC*) they might be used to confirm isolation of the desired PCR fragment. It is hypothesized that the *fliA* could be used to locate the promoter region.

Colony immunoblotting experiments **(Table 1)** were performed to demonstrate that *P. aeruginosa* flagellin protein was expressed in *E. coli* HB101 cells harboring pKW52 and pKWII. From our data ability of anti-flagellin antibodies to detect the expression of *P. aeruginosa* flagellin protein in *E. coli* cells is questionable. In support of the ability of anti-flagellin antibodies to detect *P. aeruginosa* flagellin protein in *E. coli* cells, previous

Table 1. Colony immunoblotting results of *E. coli* HB101 cells harboring pKW52 and *E. coli* INVαF' cells harboring pKWII with antiflagellin antibody. Filters with *E. coli* HB101 cells with vector pLAFR1, *E. coli* INVαF' cells with vector pCRII, PAO1 cells (Fla+), and PA103 cells (Fla-) were probed with 1:5000 dilution of the monclonal antibodies (anti-a, anti-b) and 1:1000 dilution of the polyclonal antisera (anti-1210, anti-M2). The *E. coli* host cells alone were used as negative controls in detecting antibody recognition of pKW52 and pKWII. Another control for the colony immunoblotting experiments were the uninoculated filters. Filters uninoculated with cells were probed with each primary antibody to test the ability of the secondary antibody to react with the primary in the absence of antigen.

	STRAIN	Flagellin	anti-a Mab	anti-b Mab	anti-1210 antibody	anti-M2 antibody
negative control	*E. coli* HB101 pLAFR1	—	—	—	—	++
negatve control	*E. coli* INVαF' pKWII	—	—	—	—	—
positive control	PAO1	flagellin b-type	—	+++	—	+++
positive control	PA103	No flagellar filament structure	—	—	—	+++
negative control	Goat anti-human IgG 2° Ab	—	—	—	—	—
Negative control	Goat anti-rabbit IgG 2° Ab	—	—	—	—	—

E. coli HB101 pKW52	—	—	—	—	+
E. coli INVαF' pKWII	—	—	—	—	—

Southern hybridization results were supported by the identification of primer 1N sequences in the insert of pKWII.

Colony blotting analysis of pKW52 and pKWII.

The 1.7kb flagellin a-type gene probe did not hybridize to pKW52 and pKWII. Therefore, the Southern hybridization results did not demonstrate that the flagellin b-type gene was present in clones pKW52 and pKWII. However, in this study *E. coli* cells harboring clones pKW52 and pKWII were tested for production of flagellin antigen by immunoblotting with antiflagellin antibody. Colony immunoblotting experiments would demonstrate if the flagellin b-type gene was present in clones pKW52 and pKWII despite nonhybridization of the 1.7kb flagellin (a-type) gene probe to pKW52 and pKWII.

Human monoclonal anti-a and anti-b, and polyclonal antisera anti-M2 and anti-1210 were used in the colony immunoblotting experiments. The monoclonal antibodies have been extensively tested and show a high specificity for the appropriate a or b-type antigen (Landsperger *et al.*, 1994). Also, several studies have been performed with these monoclonal antibodies and polyclonal antisera (Landsperger *et al.*, 1994; Wintenberg, 1989; Anderson *et al.*, 1989). Yet, controls were included in the colony immunoblotting experiments to validate the methodology. The PAO1 cells were an ideal positive control in these experiments, because previous results had shown that PAO1 cells were recognized by the anti-b monoclonal antibody and anti-M2 polyclonal antiserum (Landsperger *et al.*, 1994; Wintenberg *et al.*, 1989). Another positive control for the colony immunoblotting experiments were the PA103 cells. PA103 cells do not synthesize a complete flagellar filament. Anti-1210 and anti-M2 polyclonal antiserum reacted with the PA103 cells as shown by Wintenberg *et al.*, 1989. The *E. coli* host cells alone were used as negative controls in detecting antibody recognition of pKW52 and pKWII. Binding of the anti-a, anti-b, anti-1210, and anti-M2 antibodies to these cells that do not possess the a-type or b-type flagellin was not expected. Also, uninoculated filters were a negative control for the colony immunoblotting experiments. Filters uninoculated with cells were probed with each primary antibody to test the ability of the secondary antibody

to react with the primary in the absence of antigen. It was not expected that the secondary antibody would react with the primary in the absence of antigen.

E. coli HB101 cells, *E. coli* INVαF' cells, *E. coli* cells harboring cosmid pKW52, *E. coli* cells harboring plasmid pKWII, PA01 cells, and PA103 cells were grown in LB broth overnight at 37°C. The growth of the cell cultures was standardized to a 260 spectrophotometer reading of 10^9 cells per ml. The cultures were diluted to 10^5 cells per ml and a 0.1ml aliquot of the 10^5 cell dilution was spreaded onto Luria agar. The plates were incubated overnight at 37°C. The colonies were lifted from the medium with SDS treated nitrocellulose filters and then lysed with chloroform. The filters were incubated in a 1:5000 dilution of anti-a or anti-b monoclonal antibodies, or 1:1000 dilution of anti-1210 or anti-M2 polyclonal antisera. The antibody dilution of the monoclonals was determined in a pretest of the *E. coli* HB101 cells, *E. coli* INVαF' cells, *E. coli* cells harboring cosmid pKW52, *E. coli* cells harboring plasmid pKWII, PAO1 cells, and PA103 cells. Initially, the anti-a and anti-b monoclonal antibodies were diluted 1:1000. With the dilution of the monoclonal antibodies at 1:1000 binding of the antibodies was observed with the negative controls, *E. coli* host cells alone, and the controls, PAO1 and PA103 cells. The anti-a and anti-b monoclonal antibodies were diluted 1:5000. With the dilution of the monoclonal antibodies at 1:5000 binding of the antibodies was not observed with the negative controls, but recognition of the positive controls was observed. The 1:5000 antibody dilution was used for immunoblotting experiments. The anti-1210 and anti-M2 polyclonal antisera was diluted 1:1000. In the pretest of the dilution of the polyclonal antisera, binding of the antisera was observed with the PA01 and PA103 cells. The *E. coli* host cells did not react with the polyclonal antisera at the dilution of 1:1000. After washing, the filters were incubated in a 1:10000 dilution of goat anti-human or goat anti-rabbit IgG horseradish peroxidase conjugated antibody. Following rinsing the filters were developed with DAB solution. All colony immunoblot results are displayed in **Table 1**. The colony immunoblotting results of this study were determined by comparison of the immunoblot results of each clone with the control blots. In this manner, visual examination of the filters were made to determine binding of the antibodies to the clones.

E. coli cells harboring clones pKW52 and pKWII were tested for production of flagellin antigen by immunoblotting with human anti-a

monoclonal antibody. No reaction was observed with *E. coli* cells harboring clones pKW52 and pKWII. In addition, no reaction was observed with the *E. coli* host cells alone, the PAO1 cells, PA103 cells, and the uninoculated filters. No binding of the human anti-b monoclonal antibody was observed with *E. coli* cells harboring clones pKW52 and pKWII. The positive control, PA103 cells, and the negative controls, *E. coli* host cells alone, were not recognized by the anti-b monoclonal antibody. In addition, no reaction was observed with the uninoculated filters. As expected, the human anti-b monoclonal antibody did recognized the PAO1 cells. The anti-b monoclonal antibody did react with *E. coli* cells harboring clones pKW52 and pKWII, and PA103 cells at a pretest dilution of 1:1000.

E. coli cells harboring clones pKW52 and pKWII were tested for production of flagellin antigen by immunoblotting with rabbit anti-1210 polyclonal antiserum. No reaction was observed with the *E. coli* cells harboring pKW52 and pKWII, PAO1 cells, and the PA103 cells. In addition, no antibody reaction was observed with the *E. coli* host cells alone and the uninoculated filters. *E. coli* cells harboring clone pKW52, PAO1 cells, and PA103 cells did react with the rabbit anti-M2 polyclonal antiserum. In addition, reaction of the rabbit anti-M2 polyclonal antiserum was observed with the *E. coli* HB101 cells with the cosmid vector pLAFR1. There was a discernible difference in recognition of the *E. coli* HB101 pLAFR1 cells and *E. coli* cells harboring pKW52 by the anti-M2 polyclonal antiserum. The anti-M2 antiserum recognized *E. coli* cells harboring pKW52 at a lower level of binding than *E. coli* pLAFR1 cells. No binding of the antibody was observed with the *E. coli* cells harboring clone pKWII, *E. coli* INVαF' host cells, and the uninoculated filters. The immunoblot results of *E. coli* cells harboring clone pKW52 indicate that expression of flagellin antigen is detected. The immunoblot results of *E. coli* cells harboring pKWII indicate that expression of flagellin antigen is not detected.

CHAPTER 4

DISCUSSION

Summary of the results.

A brief summary of the thesis results will be made to clarify and condense conclusions of the research for the purposes of discussion.
Therefore:

1. The PAK flagellin (a-type) gene probe did not recognize sequences of clones pKW52 and pKWII. In addition, probe 1N did not recognize sequences of clone pKW52. Therefore, the Southern hybridization experiments suggest that the sequences of the PAK flagellin a-type gene, and clones pKW52 and pKWII are not complementary.

2. The reaction of *E. coli* cells harboring clone pKW52 with the anti-M2 polyclonal antiserum suggest that the PAO1 flagellin b-type gene is probably present in clone pKW52. However, the lack of recognition of *E. coli* cells harboring clone pKWII with the human monoclonal anti-b and anti-M2 polyclonal antiserum demonstrate that clone pKWII does not contain the flagellin b-type gene.

3. There is sequence similarity (70.0% - 72.5% in bases 934-972) between the PCR amplified insert of clone pKWII and the PAK flagellin a-type gene. The nucleotide sequence similarity is not significant, because the best or "perfect" matches obtained in nucleotide searches match the sequences of the insert of pKWII with those of the bacteria *Escherichia coli* and *Haemophilus influenzae*. Sequence analysis of the insert of clone pKWII demonstrated that the insert was amplified by primer 1N. Thus, PCR amplification of clone pKW52 sequences did not result in the amplification of the target sequences (flagellin b-type gene).

The PCR method with primers 1N and 1C were used to amplify the flagellin b-type gene in clone pKW52. As shown in **Figure 3**, PCR amplification yielded two fragments approximately 1.3kb and 600bp in size. The presence of two fragments indicates that the primers annealed and amplified more than one site on clone pKW52. This observation is of considerable importance. The results presented are actually a reflection of the nonspecificity of primers 1N and 1C for pKW52, because ideally, PCR amplification of DNA sequences would yield one specific product. The nonspecificity of primers 1N and 1C for pKW52 was demonstrated in the sequence analysis of the insert of pKWII which indicated that primer 1N annealed to pKW52 twice and amplified the insert. Primer 1C was not identified in the sequences of the insert of pKWII. With PCR amplification, it is expected that both primers simultaneously anneal to the template (pKW52) and amplify a single fragment.

PCR should be a suitable method to locate the flagellin b-type gene, because it is a rapid procedure and requires small amounts of DNA sample. PCR is suggested as a method for future experiments to locate the PAO1 flagellin b-type gene in clone pKW52. However, the PCR experiments described should have included additional controls. These experimental controls would enhance the identification of nonspecific PCR products by agarose gel electrophoresis of the PCR reaction samples. For example, PCR reactions would contain reaction samples with either 1 primer per tube or 2 primers per tube. After PCR amplification, the amplification products would be viewed on an agarose gel stained with ethidium bromide. The fragments resulting from amplification by one primer would have to be identified in the PCR reaction samples containing two primers. The amplification of a fragment by only one primer would demonstrate that primer's nonspecificity for the template. Sequence analysis of the insert represents another method to determine the nonspecificity of the primers for the template. The nonspecificity of the primers may be determined by identifying the location of the primers in the sequence of the insert.

Southern hybridization was a second procedure used to identify the flagellin b-type gene DNA fragments. Previous studies have shown that the N- terminal amino acid sequences of the PAK flagellin a-type gene and the

colony immunoblotting experiments have demonstrated polyclonal antisera recognition of the flagellin protein (a- and b- type) in *E. coli* cells (Wintenberg *et al.*, 1989; Totten *et al.*, 1990). In this research the *E. coli* cells were lysed with chloroform. With chloroform lysis clones pKW52 and pKWII are exposed to the anti-flagellin antibodies. To maintain the reliability of the colony immunoblotting results the colony immunoblotting experiments contained positive and negative controls **(see Table 1)**, and were standardized with regard to antibody dilution and cell counts. The colony immunoblotting experiments were analyzed at least in duplicate (Ausubel *et al.*, 1988). Assessment of the clones by a second method, colony immunoblotting, became necessary since the Southern hybridization results did not demonstrate the presence of the flagellin b-type gene in clone pKW52.

In this study, the immunoblot results of *E. coli* cells harboring clone pKW52 detected the expression of flagellin antigen. The detection of flagellin antigen was observed with the rabbit anti-M2 polyclonal antiserum. The detection of flagellin b-type antigen with the polyclonal antiserum suggests that several flagellin antigenic epitopes may be detectable with the polyclonal, because there was no detection of the flagellin b-type antigen by a specific anti-b monoclonal antibody. This may reflect specificity of a flagellin epitope recognized by the monoclonal. Nondetection of flagellin antigen by monoclonal and polyclonal antibodies with *E. coli* cells harboring pKWII may be explained as: (1) the 600bp amplified fragment may not be inserted in pKWII in the correct orientation for expression, (2) the complete fragment (flagellin b-type gene is approximately 1.7kb) for reactivity with the antibody may not be present in the clone, or (3) the 600bp fragment may not be the flagellin b-type gene.

The sequencing of the insert of pKWII **(Figures 8, 9)** was an important goal of this thesis research. The Southern hybridization and colony immunoblotting results suggest that either: 1) the flagellin b-type gene was not subcloned in pKWII, or 2) the sequences of the PAK flagellin a-type gene and PAO1 flagellin b-type gene are not highly conserved. However, Farinha *et al.*, 1993 used the *Pseudomonas aeruginosa* PAK flagellin (a-type) gene to map the location of the PAO1 flagellin b-type gene. Sequence analysis of the insert of pKWII correlated with the Southern hybridization and colony

immunoblotting results which indicated that the PCR amplification of pKW52 did not result in the amplification of the PAO1 flagellin b-type gene. The identity of the sequences of clone pKW52 amplified by primer 1N were investigated. Database sequence searches matched the sequences of the insert of pKWII with *Escherichia coli* and *Haemophilus influenzae* sequences. Interestingly, the insert sequences of clone pKWII were matched with the *E. coli rpnB* gene and open reading frame, and strain K-12 chromosomal DNA sequences. *RpnB* encodes the M1 RNA component of *E. coli* RNaseP. It is postulated that the open reading frame is involved in gluconate metabolism or a related pathway (Komine *et al.*, 1991). The *rpnB* gene and the open reading frame are in the library derived from *E. coli* strain K-12 W3110 (Kohara *et al.*, 1990). Unfortunately, the specific sequences of *H. influenzae* which matched with the insert sequences of pKWII were not placed in the BLAST database files. Based on the nucleotide matches, it is hypothesized that the pKW52 sequences amplified by primer 1N were not sequences of the cosmid pLAFR1. It is postulated that based on the match of the *rpnB* and open reading frame sequences with the insert of pKWII that sequences complementary to *E. coli* were cloned into pKW52, and were amplified by primer 1N.

In conclusion, the initial goals of subcloning, sequencing, and characterizing a subcloned fragment of pKW52 were accomplished. However, taken together the data indicate that the subcloned fragment was not the flagellin b-type gene of *P. aeruginosa* strain PAO1. Oligonucleotides and double-stranded DNA probes in the Farinha *et al.*, 1993 study may also be used in Southern hybridization experiments to locate and identify the flagellin b-type gene in clone pKW52. An alternative approach to locate and identify the flagellin b-type gene in clone pKW52 might utilize oligonucleotides as those probes used in the Farinha *et al.*, 1993 research. Since polyclonal antibody gave consistent recognization of flagellin in *E. coli* the colony immunoblotting experiments may be used to identify flagellin production by clones.

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